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Effect of Discontinued Estrogenic Stimulation upon the Development and Growth of Testicular Tumors in Mice [1]

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This paper reports the results of a study designed to investigate the biologic and histologic properties of induced testicular tumors in strain BALB/c mice. The reader is referred to publications by Gardner (*1*), Firminger (*2*), and Gardner, Pfeiffer, Trentin, and Wolstenholme (*3*) for excellent reviews of the literature dealing with this type of induced tumor. Burrows (*4*), Gardner (*5*), Bonser and Robson (*6*), Hooker, Gardner, and Pfeiffer (*7*), Shimkin, Grady, and Andervont (*8*), Hooker and Pfeiffer (*9*), Bonser (*10,11*), Gardner (*12*), and Gardner and Boddaert (*13*) observed interstitial-cell hyperplasia or interstitial-cell tumors of mouse testes following administration of estrogens. All these investigators described the gross and histologic appearance of the lesions. Bonser and Robson (*6*) also included a good review of earlier literature on interstitial-cell hyperplasia, while Shimkin, Grady, and Andervont (*8*) and Hooker and Pfeiffer (*9*) gave detailed descriptions of the microscopic sequence of events leading to the formation of tumors.

Various investigators (*6,9–12,14,15*) have transplanted induced interstitial-cell tumors into appropriate strains of mice. A survey of these efforts reveals that a total of 21 tumors were transplanted and of these, 9 failed to grow in untreated hosts, 11 grew in estrogenized hosts or in intact females and castrated males, whereas only 1 grew in untreated mice of both sexes. This last tumor was described by Bonser (*11*), who found that in the first transplant generation it grew in untreated females and estrogenized males but failed to grow in untreated males. In the second generation, however, it grew in normal males as well as in females. Jull (*16*) studied this tumor, after it had been transplanted for 8 to 10 years, and found that the latent period was dependent upon the hormonal environment.

Gardner (*12,14*) has carried out the most thorough study of transplantation characteristics of these tumors. He found that transplants that do not grow in normal hosts can lie dormant for 51 to 204 days and then start to grow following administration of estrogen to the animals.

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He also observed that transplanted tumors, after attaining an appreciable size in estrogenized hosts, may continue to grow after the stimulus has been removed. Furthermore, in personal communications (*2,16*) he stated that tumors formerly dependent upon estrogen-treated hosts for successful transplantation were now capable of growth in untreated mice, and in a recent review (*3*) wrote that ". . . after serial passage through 10 or more generations interstitial-cell tumors acquire the capacity to grow in untreated male or female hosts." Gardner's findings indicate that induced interstitial-cell tumors, although very dependent upon estrogenized hosts for early passages, show, during the course of serial passages, a strong tendency to become independent.

Hummel (*17*) found a spontaneous interstitial-cell tumor in a testis of an 18-month-old BALB/c mouse, which grew equally well, upon transplantation, in normal mice of either sex. This tumor, unlike the induced growths, was apparently independent in the first transplant generation.

The ability of induced testicular tumors of the mouse to acquire independence from hormonal stimulation upon serial passage suggested their use in studies of conditioned and autonomous tumors (*18*) and in tumor progression (*19*). In view of this property of the transplanted growths, together with the induction of primary tumors following prolonged or continuous estrogenic stimulation, it was decided to investigate the effects of discontinuing hormonal stimulation upon (1) the occurrence of induced tumors and (2) the growth of primary induced tumors.

Testicular tumors in mice are an excellent experimental material for such studies because they are easy to detect, their growth can be followed simply and accurately, and removal of a tumor-bearing testis can be accomplished with a minimum of difficulty. Moreover, histologic sections of the entire gland can be prepared, thereby enabling a study of the sequence of events occurring before and after the removal of hormonal stimulation. Stilbestrol-cholesterol pellets were used to supply estrogenic stimulation. They are easy to prepare and to administer and can be detected and removed with little difficulty from the subcutaneous tissues.

The results of this study revealed that some primary induced tumors continued to grow when the hormonal stimulus was removed. This finding led to the transplantation of testicular tumors from some mice bearing a stilbestrol pellet and from others showing continued tumor growth after removal of pellets. Many of the latter tumors proved to be independent of hormonal stimulation for growth in new hosts. In mice with regressed primary induced tumors recurrence of tumors was observed, usually within a few weeks, after receiving a second pellet. Also, some mice developed gross primary interstitial-cell tumors *after* their pellets had been removed. These findings suggested studies on the effects of administering a second pellet long after the first had been removed. Mice receiving a second pellet under these conditions developed tumors promptly, and histologic examination of testes from mice whose pellets had been removed months previously showed areas of persistent interstitial-cell hyperplasia or changes.

Materials and Methods

Male mice of strain BALB/c (*20*) were used because previous experience had shown that they developed testicular tumors following administration of a stilbestrol-cholesterol pellet (*8,21*). Unless stated otherwise, all were 2 months old when they received a pellet. These mice are hardy and long-lived but revealed 3 disadvantages as work progressed. First, within a few months after receiving 10 or 20 percent stilbestrol pellets, 5 to 10 percent of the animals died; no study was made of the cause of death. Second, an appreciable number developed leukosis, usually confined to the thymus gland, before the occurrence of testicular tumors. Third, although separated from females when 1 month of age, some males at the age of 6 to 12 months became pugnacious and fought cage mates. When this occurred, an effort was made to locate the offenders and separate them from other mice. This tendency is troublesome when a large number of mice are used in an experiment because separate cages for fighting mice occupy considerable space. Stilbestrol was not responsible for this fighting characteristic, since untreated BALB/c males showed the same tendency. These causes of death in estrogen-treated BALB/c males indicate that a death rate of approximately 25 percent is to be expected in a long-term experiment.

All animals were given an unlimited supply of Purina laboratory chow and tap water and kept under similar environmental conditions. They were maintained 8 to a plastic cage, 28 cm. long, 17.5 cm. wide, and 12 cm. deep, on a bedding of pine sawdust and cedar shavings.

Stilbestrol-cholesterol pellets were prepared according to a published technique (*22*); each was approximately 3 mm. long and 1.75 mm. in diameter and weighed approximately 5 mg. They were inserted into the subcutaneous tissues at the desired location by means of a 12-gauge trocar. Removal was easily accomplished by making a slit in the skin just over the pellet, which often extruded itself. When subcutaneous tissue adhered to the pellet, it was removed with the pellet by cutting the underlying tissues with fine scissors. No ill effects accompanied removal of pellets; anesthesia was unnecessary.

Following insertion of pellets the mice were examined at 7- to 10-day intervals for the occurrence of enlarged testes. After a tumor was detected, the testis was measured at monthly intervals and individual records were kept of each testis. Enlarged testes were removed through a scrotal incision after the spermatic cord had been tied off; no fatalities occurred after this simple surgical procedure.

Infection of the preputial gland was a common occurrence in the experimental animals. This condition was observed closely to ascertain whether it was related to the appearance of tumors, but it appeared to arise independently of testicular tumors. Infected glands were usually treated by puncture and drainage by pressure, a procedure that was seldom followed by recurrence of infection.

With the exception of a small number of mice that died while bearing large tumors, all animals were autopsied, the testes cut longitudinally

and fixed in Tellyesnicky's fluid (2 parts 40% formaldehyde solution, 20 parts 70% alcohol, and 1 part acetic acid), blocked in paraffin, cut, and stained with hematoxylin and eosin. Normal-size testes were treated in the same way after they were hardened in fixative for a few hours. All findings presented here were supported by microscopic examination.

Experimental Procedures and Results

The first experiment in the study was the administration of pellets to 2 series of 2-month-old mice. Those in the first series were permitted to carry their pellets until the occurrence of testicular tumors, and the second had their pellets removed at 4, 8, 16, 20, 24, or 28 weeks after implantation and while their testes were of normal size. The first series was to supply tumors to ascertain the effect of discontinuation of hormonal stimulation upon established tumors, and the second was to supply information on the influence of limited hormonal stimulation upon the production of tumors. The findings are presented according to these 2 lines of approach.

Removal of Hormonal Stimulus after Occurrence of Testicular Tumors

The occurrence of testicular tumors in BALB/c male mice following implantation of stilbestrol-cholesterol pellets is summarized in table 1. In addition to these animals, 87 others from the second series of mice developed testicular tumors before the time they were to have their pellets removed; all belonged to the groups that retained their pellets for 20, 24, or 28 weeks.

Data in table 1 show that many mice developed bilateral testicular tumors. Some of these arose simultaneously and others at different times. When they occurred at different times, the age of the mouse when the first tumor arose was used to compute the average age of occurrence. Groups 1 and 2 are most suitable for determining the incidence of bilateral tumors because these animals were permitted to carry pellets after the appearance of their first tumor, while many mice of groups 3 and 4 had their pellets removed as soon as they developed a testicular tumor. The first 4 groups also reveal variations in the number of mice that died without developing testicular tumors. In group 2, only 1 of 18 died—at 18 months of age—whereas in group 4, 15 of 49 died at a mean age of only 9 months. These deaths were attributable to the 3 causes discussed previously. Totals for the first 4 groups are given because these animals serve as a reference point throughout the study and are included in subsequent tables.

The last 4 groups are of some particular interest. Group 5 received 10 percent stilbestrol pellets and showed a tumor incidence of 58 percent. Of the 22 mice that failed to develop tumors, only 7 lived to the age of 17 to 18 months, when the experiment was terminated, and 10 died at 2 to 9 months of age. Thus, 10 percent pellets are effective in evoking tumors, but some mortality exists even at this dose level of estrogen. Group 6 shows that 5 percent stilbestrol pellets are not very effective in

TABLE 1.—*Occurrence of testicular tumors in strain BALB/c mice following subcutaneous implantation of stilbestrol-cholesterol pellets at 2 months of age*

Group No.	Stilbestrol in 5 mg. pellet (*percent*)	Number of mice	No. with 1 testicular tumor	No. with bilateral testicular tumors	Total no. with testicular tumors	Tumor incidence (*percent*)	Average age (*months*)	No. dead without testicular tumor	Average age at death (*months*)
1	20	26	5	17	22	85	10	4	12
2	20	18	5	12	17	94	8	1	18
3	20	71	36	19	55	77	9	16	8
4	20	49	24	10	34	69	8	15	9
Totals	20	164	70	58	128	78	9	36	9
5	10	53	24	7	31	58	10	22	11
6	5	46	6	1	7	15	13	39	14
7	20*	54	15	8	23	43	10	31	12
8	20†	40	0	0	0	—	—	40	14

*Previously these pellets had been in other mice for 16 weeks.
†Previously these pellets had been in other mice for 15 to 22 months

producing tumors: Survivors of this group were killed when they were 17 to 18 months old. The last 2 groups show the loss of activity when 20 percent pellets resided in the subcutaneous tissues of other hosts before implantation into 2-month-old males. Group 7 showed an incidence of 43 percent, which was lower than any of the first 4 groups. None of group 8 developed a testicular tumor, and only 6 mice died before the age of 7 months. Mice of groups 7 and 8 were killed when they were 17 months old. Apparently 20 percent stilbestrol pellets lose their tumor-producing ability after 15 to 21 months' exposure to the subcutaneous tissues.

Data in table 1 confirmed earlier findings that implantation of stilbestrol-cholesterol pellets into strain BALB/c males elicits a high incidence of interstitial-cell testicular tumors (*8,21*). Twenty percent stilbestrol pellets induced tumors in 78 percent of mice at an average age of 9 months, or within 7 months after insertion of pellets. With 10 percent pellets, the testicular-tumor response occurred in 58 percent at a mean age of 10 months, and with 5 percent pellets only 15 percent of the mice developed tumors, at an average age of 13 months.

In conformity with the purpose of the study, pellets were removed from mice that had developed testicular tumors to ascertain the response to discontinued hormonal stimulation. The findings are shown in table 2, where they are listed according to the length of the testes when the pellets were removed. There was a rough correlation between the size of the testes and the rate of regression: 13 of 19 tumor-bearing testes that were 9 mm. in length regressed, whereas only 1 of 8 that measured 14 to 16 mm. did so. Approximately 50 percent of testes measuring 10 to 12 mm. receded to normal size. After excision of pellets, the size of the testes reflected pronounced variations in response to the termination of estrogenic stimulation. Growth or regression was rapid in some and slow in others. Some receded to almost normal size and then resumed progressive growth, while others continued to grow for varied periods of time and then regressed to normal size. Bilateral tumors gave interesting contrasts, for in some mice one testis enlarged progressively while the other regressed. The result of this part of the study indicated that some induced testicular tumors were independent of, and others dependent on, hormonal stimulation for continued growth. Independence was related to the size of the

TABLE 2.—*Response of testes to removal of pellets*

Length of testes* (*mm.*)	Number of testes	Testes that continued to enlarge (*number*)	Testes that regressed to normal size (*number*)	Incidence of regressions (*percent*)	Time to regression (*days*)	
					Average	Range
9	19	6	13	68	28	7–50
10	37	19	18	49	75	7–218
11	35	13	22	63	64	30–155
12	46	29	17	37	93	27–218
13	30	20	10	33	133	36–209
14–16	8	7	1	12	78	—

* Length of normal testes is 6 to 7 mm.

TABLE 3.—*Recurrence of testicular tumors in mice receiving a second stilbestrol-cholesterol pellet*

Time between regression of testes and implantation of second pellet (*months*)	Number of mice	Results:— Testicular tumor / No testicular tumor (*time in months*)						Totals
		1	2	3	4	5	6	
2	2	2/0						2/0
3	3	3/0						3/0
4	14	9/0	2/1	1/1				12/2
5	21	18/0	2/(	0/1				20/1
6	5	5/0						5/0
Totals	45	37/0	4/1	1/2				42/3
Controls*—no pellet	46	1/3	6/5	1/7	1/11	0/5	1/5	10/36
Controls†	164			1				1

*These mice are controls for spontaneous recurrence of tumors following regression of testes to normal size after removal of pellets.

†These animals show the occurrence of tumors during the first 6 months after implantation of first pellets at age of 2 months; *see* table 1.

tumor, in that a greater proportion of larger tumors continued to grow, and a greater proportion of smaller tumors regressed, with the removal of estrogenic stimulation.

Regression of testes to normal size in a number of animals presented an opportunity to observe the rate of tumor recurrence and the response of testes to a second hormonal stimulus. Mice in which testes had receded to normal size received pellets containing 20 percent stilbestrol. The results are presented in table 3, in which the mice are divided into groups according to the elapsed time between regression of testes and implantation of second pellets. Of 45 mice, 37 had a recurrence of testicular tumors within 1 month after receiving pellets; some testes became enlarged within 1 week. Of 40 mice that had remained free of tumor from 4 to 6 months, 32 had recurrences within a month, while 5 others showed recurrences within the next 2 months; 3 died without recurrence. All tumors arose in testes in which a tumor had regressed.

Two kinds of controls are presented. The first consists of 46 animals in which testicular tumors recurred spontaneously after removal of the first pellet; they did not receive a second one. Because recurrences were noted in 7 of 15 mice within 2 months after their testes had receded to normal dimensions, pellets were inserted into most of the experimental animals only after their testes had remained at normal size for at least 2 months. Of 23 mice kept from 4 to 6 months, only 2 had spontaneous recurrences. These mice are comparable to the last 40 animals of the experimental series and serve to emphasize the prompt reaction of the experimental animals to hormonal stimulation a second time. However, the recurrences in 2 mice of this group of controls 4 and 6 months after regression implies that interstitial-cell hyperplasia in these cases had lost its dependence upon hormonal stimulation.

The other control animals are from those listed in table 1 and are included to reveal the rapid reaction of the experimental mice to the second pellet. Only 1 of 164 animals developed a tumor within 3 months after receiving a 20 percent stilbestrol pellet. These controls were only 2 months old when they received pellets, and the experimental animals were 12 to 14 months of age. This difference in age was not responsible for the reaction of the older animals, for in an unpublished experiment performed in this laboratory, 93 BALB/c males received 20 percent stilbestrol pellets when 12 to 14 months of age and only 3 developed testicular tumors within 3 months after insertion of pellets.

Removal of Hormonal Stimulus before Occurrence of Testicular Tumors

Data in table 3 indicate that, although the testes had returned to normal size, conditions existed and persisted in them which reacted promptly when hormonal stimulation was renewed. This observation suggested the possibility that similar conditions might be present in testes of mice that had carried pellets for limited periods of time without developing testicular tumors. A number of such animals were available from the second series of mice, those which received 20 percent pellets when 2 months of age and had the pellets removed 4 to 28 weeks later. Surviving mice from 4 groups that had lost their pellets 4, 16, 24, and 28 weeks after insertion were either given a second 20 percent stilbestrol pellet or kept as untreated controls. The experiment is summarized in table 4, in which it is seen that second pellets were implanted 5 to 12 months after removal of the first pellets. The over-all result was a tumor incidence of 34 percent in mice that received hormonal stimulation a second time and an incidence of 2 percent in untreated controls that had carried first pellets from 4 to 28 weeks. Testicular tumors arose in 50 percent of mice that had carried their first pellets for 24 or 28 weeks and received their second pellets 5 to 9 months later. Moreover, the tumors appeared within only 4 months after insertion of second pellets. Table 4 also includes the 164 controls (*cf.* table 1) that received pellets when 2 months old, of which only 12, or 7 percent, developed tumors within 4 months. These data suggest that an earlier period of hormonal stimulation had "conditioned" the interstitial tissue to react promptly to a second stimulus.

All mice of the experimental subgroups of table 4 received second pellets on the same day. The experiment was continued for 4 months and, because of the clear-cut results, it was decided to sacrifice all surviving mice in order to procure histologic sections of their testes. Sections of testes were examined without knowledge of whether the animals had received second pellets or were in the control groups.

The results of the histologic study are presented in table 5. The presence of interstitial-cell nodules or tumors in the testes of 17 of 31 mice belonging to the experimental subgroups suggests that these animals might have developed gross tumors had they been kept longer.

TABLE 4.—*Occurrence of testicular tumors in mice receiving a second 20 percent stilbestrol-cholesterol pellet*

Group	Length of exposure to first pellet (*weeks*)	Age when given second pellet (*months*)	Time after removal of first pellet (*months*)	Subgroup*	Number of mice	Occurrence of tumors after second pellet (*months*)					Tumor incidence (*percent*)	Average time for tumor development (*months*)
						1	2	3	4	Totals		
1	4	14–15	11–12	Experimental	12	—	1	—	—	1	8	2
				Control	10	—	—	—	—	—	0	—
3	16	14–18	8–12	Experimental	34	—	4	—	—	4	12	2
				Control	27	—	—	—	—	—	0	—
5	24	15–17	7–9	Experimental	42	1	6	8	6	21	50	3
				Control	35	—	—	—	1	1	3	4
6	28	14–15	5–6	Experimental	23	1	6	3	2	12	50	3
				Control	19	—	—	1	—	1	5	3
Totals				Experimental	111	2	17	11	8	38	34	3
				Control	91	—	—	1	1	2	2	4
Controls†					164	—	—	1	11	12	7	4

*Experimental mice recived a second pellet; control mice did not.
†These animals received pellet when 2 months old; *see* table 1.

TABLE 5.—*Microscopic appearance of testes from mice receiving a second stilbestrol-cholesterol pellet and from mice receiving but 1 pellet*

Group	Length of exposure to first pellet (*weeks*)	Age when given second pellet (*months*)	Age when killed (*months*)	Subgroup*	Number of mice	No. with nodular hyperplasia or small testicular tumor	No. with nodular hyperplasia	No. with interstitial-cell hyperplasia or changes	No. with normal testes
1	4	14–15	19	Experimental	7	1	3	1	2
				Control	7	—	—	—	7
3	16	14–18	19–22	Experimental	17	12	2	1	2
				Control	17	—	2	13	2
5	24	15–17	20–21	Experimental	5	3	2	—	—
				Control	22	—	4	10	8
6	28	14–15	19–20	Experimental	2	1	1	—	—
				Control	10	—	1	5	4
Totals				Experimental	31	17	8	2	4
				Control	56	—	7	28	21

*Experimental animals received second pellet. Control animals did not.

Testes of 10 other experimental mice showed lesser degrees of interstitial-cell hyperplasia or changes, and testes of only 4 animals were considered to be normal. Occurrence of definite nodular hyperplasia or small tumors only in the mice of the experimental subgroups indicates that this was a response to the stimulus supplied by second pellets.

All mice of the control subgroup that had carried pellets for 4 weeks and were killed 16 months later had normal testes. However, in the other control subgroups, of 49 mice that had carried pellets for 16 to 28 weeks and came to autopsy 10 to 16 months later, the testes of 35 showed some interstitial-cell hyperplasia or changes. This demonstrated that, in the majority of mice, the effects of estrogenic stimulation for 16 to 28 weeks were plainly visible 10 to 16 months later.

The observation that temporary estrogenic stimulation elicits changes in the testis of the mouse which last for many months raises the question whether the arrested hyperplasia can proceed to tumor formation without an extrinsic hormonal stimulus. An answer was supplied by mice from which pellets were removed before they showed gross evidence of tumors. Two such animals are included among the controls in table 4. These mice and others in which testicular tumors arose after removal of pellets are shown in table 6, which summarizes the occurrence of testicular tumors in 30 mice from 1 to 12 months following excision of pellets. No obvious technical procedure can be held responsible for these tumors. Removal of pellets was so easily performed that small pieces could not have been left in 30 mice. This part of the study is receiving further consideration, for, in the production of these tumors, hormonal stimulation required latent periods comparable to those of chemical carcinogens (*23*). The finding suggests that the estrogen initiated changes which persisted or were maintained by endogenous hormonal mechanisms within the mice.

Occurrence of tumors listed in table 6 is correlated with the age of the mice in which they appeared. Further information concerning these tumors is shown in table 7, where data on the mice are presented according to the original purpose of the study. It is seen that tumors arose in both series of experimental animals, but the incidence of tumors is relatively low because a considerable number of animals of both series were not

TABLE 6.—*Time of occurrence of testicular tumors in mice after 20 percent stilbestrol-cholesterol pellets were removed*

Age of mice when pellets were removed (*months*)	Time between removal of pellets and occurrence of tumors (*months*)											Totals
	1	2	3	4	5	6	8	9	10	11	12	
5						1			1		2	4
6										1		1
7	1	1	1	2			1	1		1		8
8	2		1	1	1	2	1	1				9
9	2		1			3						6
10		1										1
11	1											1
Totals	6	2	3	3	1	6	2	2	1	2	2	30

TABLE 7.—*Frequency of occurrence of testicular tumors after removal of stilbestrol pellets*

Series	Number of mice	Length of exposure to pellet (*weeks*)	No. developing tumor after removal of pellet	Tumor incidence (*percent*)	Time between excision of pellet and occurrence of tumors (*months*)											
					1	2	3	4	5	6	8	9	10	11	12	
Mice with pellets removed in absence of tumor....	106	16	3	3						1			1		1	
	24	20	1	4										1		
	149	24	8	5	1	1	1	2			1	1		1		
	80	28	6	8	1		1		1	2		1				
Totals.....	359	—	18	5	2	1	2	2	1	3	1	2	1	2	1	
Mice with pellets removed in presence of tumor...	118	—	12	10	4	1	1	1		3	1				1	

permitted to live their full life span. A separate experiment is needed to ascertain the incidence of tumors after removal of stilbestrol pellets.

Mice that had normal-size testes when their pellets were excised showed a rough correlation between the time they carried pellets and the occurrence of tumors, but it is seen that 3 tumors occurred in mice that were exposed to estrogenic stimulation for only 16 weeks. The 12 mice of the other series, which developed their first tumors while carrying pellets and second tumors thereafter, were of interest, since the second tumors arose in testes which were of normal size when the pellets were excised. In 5 of these animals first tumors grew progressively and second tumors arose within 2 months. Of the remaining 7 mice, the first tumor of 1 remained stationary and of 2 receded and then resumed growth, while in the last 4 animals the first tumors regressed. The occurrence of second tumors in all 12 of these mice indicates that the testes reacted independently to estrogenic stimulation, and complete regression of a tumor-bearing testis in 4 animals followed by the appearance of tumor in the other testis is evidence that the testicular tumors included in tables 6 and 7 were not induced by pieces of pellets remaining in the mice. In our experience testicular tumors did not regress while their hosts carried stilbestrol pellets.

Transplantation of Testicular Tumors

This part of the study was started when it was found that some primary induced tumors continued to grow progressively in the absence of estrogenic stimulation (table 2). Since previous investigators had found that these tumors did not grow readily in new hosts and since almost all that did grow were dependent upon estrogenic stimulation, it was decided to transplant a number of tumors—some from mice that carried pellets and others that continued to grow after removal of the pellet from the host.

A uniform procedure was followed in the transplantation of all primary tumors. The testis was removed, measured, cut longitudinally, and a piece placed in fixative. Tissue adjacent to the fixed portion was cut into small pieces that fitted snugly into a 12-gauge trocar and introduced subcutaneously into the right axillary region of strain BALB/c mice 2 to 4 months old. An equal number of males and females received the tumor implants and half the mice of each sex received a 10 percent stilbestrol pellet in the left axilla. Thus, 4 groups of mice were inoculated with each tumor, each group usually consisting of 2 animals.

Seventy tumors, 20 from mice carrying pellets and 50 from mice free of pellets, were transplanted and 56 grew in first-transplant-generation hosts. This unexpectedly high proportion of positive results led to the adoption of an arbitrary scheme to test each of the 56 tumors for its ability to acquire independence of hormonal stimulation. Four or 8 mice were inoculated for each transfer generation. When 4 were used, they consisted of 2 males and 2 females. One male and 1 female received 10 percent stilbestrol pellets subcutaneously in the left axilla immediately before inoculation; the remaining 2 mice did not receive pellets. When 8 mice were used, they were divided into 4 groups of 2 mice each. This

routine was followed through 4 transplant generations only. The only deviations were (1) in a small number of passages only male or female mice were used because mice of both sexes were not available, (2) only 2 untreated males and females were inoculated to determine whether a tumor which had grown in an untreated male had achieved autonomy; the tumors grew in almost all such mice. A tumor was discontinued as soon as it had become independent. Thus, some tumors were not carried through 4 serial transfers.

When no tumor was found within 6 months in any mouse of a first passage, the mice were killed and the tumor was considered one that failed to grow. When a tumor grew in estrogenized hosts but failed to grow in untreated hosts within 6 months, the latter animals were given a 10 percent stilbestrol pellet to ascertain, as shown by Gardner (*14*), whether the dormant transplant was capable of growth. All mice treated in this manner were kept under observation for 4 months, at which time all those without tumor were killed.

With this rigid procedure, maintained through 4 transplant generations, the tumors were classified as follows:

1) Those that failed to grow in any first-generation host.
2) Dependent tumors—those that grew only in mice carrying stilbestrol pellets.
3) Partially dependent tumors—those that grew in all untreated females, in only a few untreated males, and in all males that received pellets 6 months after inoculation.
4) Independent tumors—those that grew in all untreated females and nearly all untreated males. The few untreated males that did not grow the tumor did so promptly when given a pellet 6 months after inoculation. There were only a few such males in this group.

The results are summarized in table 8, where those tumors transplanted from mice carrying pellets are designated "group 1" and those that grew or arose in mice after removal of pellets are designated "group 2." In view of the results of earlier workers, the development of 6 independent tumors from group 1 within 4 transfer generations was surprising, and the

TABLE 8.—*Results of transplantation of 70 tumors of the testis in mice*

Group	Origin of tumors	Tumors transplanted (*number*)	Tumors that failed to grow (*number*)	Dependent tumors (*number*)	Partially dependent tumors (*number*)	Independent tumors (*number*)
1	Tumors in mice carrying pellets.	20	8	2	4	6
2	Tumors that continued to grow in mice after removal of pellets or that first appeared after removal of pellet.	50	6	2	3*	39†

* Includes 1 tumor that arose after removal of pellet.
† Includes 10 tumors that arose after removal of pellet.

observation that 39 of 50 tumors from group 2 became independent was unexpected. The primary objective of this part of the study was the acquisition of a small number of independent growths in order to compare their biologic and histologic characteristics with those of dependent tumors. This accounts for the transplantation of 11 tumors that arose after removal of pellets, of which 10 proved to be independent and 1 partially dependent. It was hoped that a few of these growths had achieved independence, but the fact that 10 of them and 29 of 39 other tumors from group 2 became independent of hormonal stimulation was far beyond the goal of the experiment. Many tumors included in table 8 were found to be independent during the first 2 passages: A summary of the number of tumors that were independent, and the generation in which they achieved independence, is presented below.

Group no.	First generation	Second generation	Third generation	Fourth generation
1	0	3	2	1
2	21	16	0	2

Of the 10 tumors arising in mice after removal of pellets, 8 were independent in the first generation and 2 in the second. These data emphasize the ability of the testicular tumors in this study to achieve independence. Attention is directed, however, to the inability of 14 primary tumors to establish themselves even in estrogen-treated hosts within a period of 6 months, and, furthermore, 6 of these tumors had continued to grow in the absence of a stilbestrol pellet.

The biologic characteristics and histologic appearance of tumors that failed to grow and those that were independent have been compared, but thus far analyses of the data have not revealed any significant differences. The tumors were compared according to the size of the primary induced tumors,[3] the time between the appearance of tumor and transplantation, and the time between excision of pellets and transplantation. In the latter analysis the average time for tumors that failed to grow was 60 days and for those that were independent it was 101 days, but a total of 6 mice in one group and 29 in the other made comparison tentative.

Considerable space could be devoted to the biologic properties of the transplanted tumors but it is believed that a brief summary of their chief characteristics will suffice for the following reasons: (1) Because of the large number of tumors undergoing transplantation, none was carried beyond the fourth transplant generation; (2) all tumors have been discontinued; (3) other such tumors can be procured easily. The most interesting characteristics, some of which are found in most transplantable tumors, are shown below.

1) As shown in table 8, tumors from mice of group 2 showed a greater tendency to independence than those of group 1. This indicates that removal of pellets aided in the selection of independent growths.

[3] The smallest was 11 × 7 × 7 mm. in size and was partially dependent: the largest was 22 × 18 × 18 mm. and was independent in the second generation.

2) There was a wide range in the latent periods (time between transplantation and appearance of tumor) of different tumors, and those that were transplanted for several generations showed decreases in latent period in succeeding generations. Eighty-five tumors were transplanted from first-generation hosts, and the average time between inoculation and transplantation of these tumors into second-generation hosts was 116 days. The fastest-growing tumor required 35 days to attain a size of 17 × 10 × 7 mm. and was in an estrogenized male. The same tumor required 54 days to reach 17 × 10 × 6 mm. in size in an untreated female. This tumor was independent in the first generation. The next most rapidly growing tumor was 18 × 12 × 10 mm. in dimensions within 39 days in an untreated female and was also independent in the first passage generation.

   The slowest-growing tumor required 265 days to become 12 × 10 × 7 mm. in an estrogenized female and was partially independent. The next slowest was from an untreated male and was 14 × 10 × 8 mm. 231 days after inoculation. This tumor was independent in the second passage generation. These examples show the range in latent periods of different tumors in first-generation hosts.
3) Nearly all tumors, including those that were independent, grew better in estrogenized mice. This characteristic was more pronounced in those that acquired independence in later passages. Of the 85 tumors transplanted from first-generation hosts, 44 grew in estrogenized animals and required an average time of 95 days between inoculation and transplantation; 41 grew in untreated animals and required a mean time of 138 days. However, in some passages, tumors arose earlier in untreated mice, and 1 tumor grew better in untreated males than in untreated females or treated males and females.
4) They retained the shape of the first-passage tumor through succeeding transfers. Some tumors remained spherical and others, round and flat. All were composed of soft, yellow-colored tissues and a few contained hemorrhagic areas.
5) Some tumors were consistently surrounded by a yellow-colored, gelatinous substance in the subcutaneous tissues. Most were confined to estrogenized hosts, but some tumors elicited this reaction in untreated hosts.
6) Some of the 14 tumors shown in table 8 that failed to grow in first-generation hosts were implanted into treated males, which developed primary induced tumors of the testis.
7) None of the tumors showed a tendency to produce metastases.

This brief summary of properties is sufficient to show that, as revealed by 4 transfer generations, induced tumors in the testes of mice are comparable to other transplantable tumors.

Results with mice that had not received pellets at the time of inoculation but did receive them 6 months later because the tumors failed to

grow deserve some comment. These findings are presented briefly because they confirmed Gardner's observations and because the animals were kept for only 4 months after receiving pellets. A total of 162 mice were used and, of 63 females, 38 developed tumors, while of 99 males, 82 developed tumors. Forty-nine of the 162 mice carried transplants from primary tumors; 33 developed tumors at a mean time of 70 days (range 23–133 days) after receiving pellets. Mice inoculated with the 14 tumors that failed to grow (table 8) are not included in this summary. Fewer tumors arose in mice during the first 2 passages than in the last 2 because the tumors were acquiring independence while undergoing serial transfer. Also, more tumors arose in mice inoculated with tumors which became independent than in those inoculated with tumors that remained dependent or became only partially dependent. Thus, under the conditions of this investigation, the ability of tumor transplants to remain dormant for 6 months and then to start growing in estrogenized hosts was largely dependent upon the inherent capacities of the primary tumor to achieve independence.

The occurrence of leukosis in the BALB/c males of this study is summarized in table 9. BALB/c mice are known to develop generalized leukosis spontaneously, but seldom before the age of 15 months. An incidence of approximately 35 percent is reached beyond the age of 15 months (*8*). The chief reason for presenting data in table 9 is to show the frequency of leukosis confined to the thymus gland in the estrogen-treated males. This type of the disease is not common in the colony from which the animals were derived but had been observed before in mice exposed to stilbestrol (*8*). In the present study, thymic enlargement was present in 38 of 407 mice that had pellets removed from 8 to 28 weeks after implantation and in 26 of 164 mice that carried pellets until they developed testicular tumors or died from other causes. Many of the latter lesions were found when the mice came to autopsy after their testes had become very large. It is known that estrogens evoke

TABLE 9.—*Occurrence of leukosis in 6 groups of strain BALB/c males exposed to 20 percent stilbestrol-cholesterol pellets for different periods of time*

Group	Time exposed to pellet (*weeks*)	Number of mice	Mice with thymic enlargement (*number*)	Average age (*months*)	Mice with generalized leukosis (*number*)	Average age (*months*)	Mice dead without autopsy (*number*)	Average age (*months*)
1	4	29	0	—	0	—	8	12
2	8	48	2	15	0	—	4	10
3	16	106	8	14	3	19	28	10
4	20	24	2	12	2	17	6	12
5	24	149	15	13	4	15	46	10
6	28	80	11	10	5	12	17	9
Totals		436	38	13	14	15	109	10
Controls*		164	26	8	4	10	21	9

* Controls carried pellets until tumor or death.

leukemia in mice of many strains (*1*). Hence, the results of this study suggest that stilbestrol hastened the appearance of leukosis and, in addition, appeared to localize it in the thymus gland.

Another interesting outcome shown in table 9 is the large number of animals that died and were not autopsied because of decomposition. Of 600 mice used, 131, or 22 percent, died without autopsy. This was far above the number of such animals in most experiments performed in this laboratory, and it is reasonable to assume that some deaths were attributable to tracheal compression by an enlarged thymus.

Histologic Observations

There are available in the literature a number of thorough descriptions of the morphology and histogenesis of interstitial-cell tumors of the testes in mice. A brief recapitulation is given here for orientation. Essentially, however, the observations are identical to those already recorded for BALB/c mice (*8*) and appear to be equally applicable to strain A mice (*9*), except for some differences in quantity and time relationships.

Figures 1 and 2 are cross sections of normal testes and of bilateral tumors. The histologic appearance of the normal testis is shown in figures 3 and 4.

Figures 5 and 6 present the earliest changes in the appearance of interstitial-cell elements in animals receiving estrogens: increase in the interstitial area, topographic disorientation of the identifiable Leydig cells, and the appearance of large brown cells that are phagocytic. These changes are usually found within 3 months after the administration of estrogen to strain BALB/c mice and, apparently, somewhat later in strain A mice. The changes recorded in figures 5 and 6, however, are from a testis removed 13 months following the cessation of estrogen administration, which was given for 16 weeks. It indicates the long persistence of such changes and suggests that the changes are irreversible yet incomplete for further progression toward a tumor. The persistence of the large brown cells is of particular interest and would favor the interpretation of such cells as altered Leydig cells rather than as foreign mesenchymal elements. This finding is at some variance with the descriptions of Hooker and Pfeiffer (*9*), who state that the brown cells and other interstitial-cell changes disappear in strain A mice 5 months after the termination of a 6-month period of weekly injections of estrogens in oil.

Figures 7 and 8 record a somewhat more advanced stage of interstitial-cell hyperplasia, with the formation of a small nodule between the spermatic tubules. This area had persisted for 15 months following the removal of estrogenic stimulation. As given in table 5, changes of this type, or of the type pictured in figures 5 and 6, were observed in 15 of 17 mice that had no estrogenic treatment for at least a year following a 16-week exposure to a subcutaneously implanted pellet of stilbestrol. As table 7 shows, 3 of 106 mice that had stilbestrol pellets implanted for 16 weeks and then removed developed gross testicular tumors. This in-

dicates further that most of the alterations in the interstitial-cell areas were both irreversible and nonprogressive.

Grossly, testes containing persistent microscopic alterations could be separated into 3 groups: those resembling normal testes; those of normal size and color but containing 1 or a few small yellow surface spots; those of normal or slightly subnormal size but distinctly gray in color.

The hyperplastic interstitial-cell areas progressed through formation of nodules to actual tumors. The distinction between nodule and tumor is entirely one of subjective determination. Previously, the term "tumor" was restricted to masses of a size double that of a normal testis (*8*); in the present study, nodular areas occupying one third to one half of the testis were considered as small tumors. Since there were no discernible differences in the appearance of the individual cells or the topography of the small and the large masses, the distinctions are arbitrary and of no importance in comparison with the biologic property of continued growth.

The histologic appearance of the tumors is presented in figures 9 to 12. The masses are composed of solid sheets or cords of large, polygonal cells, supported by a sparse stroma. Figure 10 clearly shows the extensive, delicate endothelial-lined spaces containing blood, around which cells resembling Leydig cells are arranged in adenomatous fashion. Hemorrhage was a frequent and extensive feature in the larger tumors. Figures 11 and 12 show a morphologic variant, often seen in the tumors, of smaller, more fusiform cells with darker nuclei. This variant is referred to as "third generation" cells by Hooker and Pfeiffer (*9*), an unfortunate term that implies the filial descent the authors disclaim. Although these cells have more variation in nuclear size and shape than the larger Leydig-like cells, mitotic figures are equally difficult to find.

Even in the larger tumors, some remnants of the spermatic elements, in the form of atrophic tubules, usually could be found, compressed between the tumor and the testicular capsule. In 10 instances there was definite histologic evidence of the penetration of the capsule by the tumor.

Although there was no systematic search for metastases, distant foci of interstitial-cell tumors were found in 5 animals. In 3 the growth was epirenal in location, as described previously (*8*) and as pictured in figures 13 and 14. Hooker and Pfeiffer (*9*) believe that this represents metastasis to lymph nodes located in this area. The present material was not satisfactory for the examination of this point, except to note that in the 3 large masses observed there was no clear evidence of the presence of lymph-node structure or of remnants thereof. In 2 additional animals, tumor deposits were found in the omentum, separate and distant from the primary growths. One of these intra-abdominal metastases is given in figure 15.

The design of this study allowed observations on testicular tumors that receded to the normal size of testes following withdrawal of estrogen (table 2) and on testicular tumors that arose rapidly in such receded masses upon the resumption of estrogenic stimulation (table 3).

In some instances of testicular enlargement that receded following the

removal of the stilbestrol pellet, the testes eventually became small, hard, yellow masses with calcified capsules. Occasionally, no testicular elements or interstitial-cell tumor could be identified histologically, the mass consisting of a fibrous tissue with calcification. In most instances, however, there was clear evidence of the preservation of a tumor node within the atrophic testis. Two such examples are given in figures 16 and 17. Both show almost complete destruction of normal elements and capsular thickening and calcification, yet there is present viable tumor tissue composed of the same type of cells as found in the usual interstitial-cell tumors of mice. Even in the almost entirely destroyed testis pictured in figure 18, there were small foci of cells resembling the large brown, cells that are a characteristic finding in the earlier stages of interstitial-cell hyperplasia.

The interstitial-cell tumors that arose in atrophic testes upon the resumption of estrogen administration were in no wise distinguishable from the usual tumors, except for the presence of calcification at the capsular periphery of such masses.

Comparison and attempted differentation on the basis of morphologic characteristics between tumors that transplanted successfully and those that did not were unrewarding. Thus, no morphologic parallel with the biologic characteristics of dependence and independence could be demonstrated. It should be pointed out that the observations were limited to the examination of 1 to 3 longitudinal sections of testes and to preparations stained only with hematoxylin and eosin.

Examination of transplants of testicular tumors showed excellent preservation of all features of the primary tumors up to the 4th generation of transplantation, to which this study was limited. As in the primary tumors, the most common cells resembled the Leydig cells, growing in sheets or cords, with a sparse stroma. Areas of hemorrhage were encountered as often as in the primary tumors. The growth of the transplants appeared to be indolent, with rare mitotic figures and little evidence of acute invasion of contiguous structures. The tumors appeared to grow along muscle planes, and in several instances surrounded muscle bundles, lymph nodes, and blood vessels without evidence of penetration. Attempts to show morphologic characteristics indicative of dependence or independence from estrogenic stimulation of the host were as unsuccessful with the transplants as with the primary tumors.

As indicated in table 9, other than testicular tumors, the most common neoplastic condition in BALB/c mice was a leukosis involving primarily the thymic gland. The process occurred in approximately 12 percent of the mice at an average age of about 1 year. The histologic appearance of the lesion is that of a small cell lymphosarcoma.

Figures 19 to 22 present a curiosity—a metastasis of a primary pulmonary tumor to a testis containing an interstitial-cell nodule, the remnant of a tumor that had regressed following the removal of the stilbestrol pellet.

Discussion

The work presented here does not require lengthy discussion. It was undertaken to investigate the properties of induced interstitial-cell tumors of the testis because earlier investigators had found that the development and transplantation of these tumors were dependent upon estrogenic stimulation. Tumors of the mouse testis could be useful in the study of dependent tumors because of the ease of detection and the opportunity to follow the rate of growth. Furthermore, the size of a mouse testis makes possible histologic studies of the entire gland and its relationship to the tumor it contains.

It was known from earlier experiments that BALB/c mice developed testicular tumors following exposure to stilbestrol pellets. Removal of these pellets made it possible to study the influence of the discontinuation of hormonal stimulation on the growth of primary induced tumors as well as to ascertain the influence of limited hormonal stimulation upon the subsequent development of tumors.

The investigation confirmed early findings that subcutaneous administration of stilbestrol-cholesterol pellets produced a high incidence of interstitial-cell testicular tumors, many of which were bilateral, in strain BALB/c mice. Estrogen also induced the earlier appearance of leukosis, which was confined chiefly to the thymus gland.

Removal of pellets from mice bearing testicular tumors proved useful for determining whether the tumors were dependent upon further estrogenic stimulation for progressive growth. When pellets were removed from mice with testes measuring 10 to 12 mm. in length, approximately half regressed to normal size within varied periods of time, while the remainder continued to enlarge. This finding revealed a remarkable range in the biologic behavior of these tumors; in some mice with bilateral tumors, one continued to grow while the other regressed.

When mice whose testes had receded to normal size, and remained so for 2 to 6 months, were given a second stilbestrol-cholesterol pellet, their tumors recurred promptly. This finding has been confirmed with mice whose testes had remained at normal size for 6 months (*24*). It is clear that regression in the size of a testis does not imply disappearance of interstitial-cell hyperplasia or tumor. Further evidence of lasting changes was supplied by the spontaneous recurrence of tumors in mice that had not received a second pellet. This observation has been extended by keeping mice for 6 months after their testes were of normal size and, thus far, these animals show a 50 percent incidence of spontaneous recurrences at an average time of 3.5 months (*24*). Prompt recurrence of tumors following additional hormonal stimulation together with the tendency of tumors to recur spontaneously present an opportunity to explore the possibility of preventing such recurrences. The influence of androgens in this respect is receiving consideration.

The observation that interstitial-cell hyperplasia persisted in testes which had receded to normal dimensions suggested that similar conditions might be present in testes of mice whose first pellets had been removed

in the absence of any gross evidence of testicular enlargement. When mice that had lost their first pellets 5 to 9 months earlier received another pellet, 50 percent developed testicular tumors within 4 months. This strongly suggested the persistence of changes within the testes of mice that had lost their pellets months previously. To ascertain whether these changes were detectable microscopically, all surviving mice that had been exposed to but 1 pellet were killed and their testes examined microscopically. Interstitial-cell hyperplasia or changes were found in many animals that had carried pellets for 16 to 28 weeks. These changes had persisted for 10 to 16 months after excision of pellets and, as shown by the occurrence of testicular tumors in litter mates that had received second pellets, these changed tissues were capable of reacting promptly to a second hormonal stimulation. This finding has been confirmed with mice that carried first pellets for 8 to 16 weeks and received second pellets 6 months later (*24*).

This part of the study presented a number of interesting problems and permitted 2 general conclusions. The first is that limited hormonal stimulation initiated changes in the testes which persisted for a long time. These changes, which were detectable by microscopic examination, suggest that induced interstitial-cell tumors of the testes of mice are the end product of a gradual process. The second conclusion is based upon the prompt reaction to stimulation a second time. The appearance of testicular tumors under these conditions suggests that they were the result of several insults experienced by the interstitial cells of the testes.

Another observation of interest was the appearance of testicular tumors in mice that had lost their pellets from 1 to 12 months previously. Such results indicated that the occurrence of all tumors of the testis in BALB/c mice was not dependent upon prolonged or repeated extrinsic estrogenic stimulation. Apparently interstitial-cell hyperplasia initiated by exposure to hormonal stimulation proceeded to malignancy in the absence of any known further stimulation. This finding has been confirmed but much more work is essential to elucidate the mechanisms responsible for the appearance of these tumors. Establishment of a technique assuring a reproducible incidence of the tumors may be the first step toward the solution of this problem.

Transplantation of testicular tumors gave unexpected results. Seventy tumors were transplanted for the purpose of procuring only a few that were independent of estrogenic stimulation for progressive growth, but 45 were able to grow in untreated males within 4 transplant generations. This phase of the study showed that the pellet technique is satisfactory for the induction of testicular tumors which are independent of further estrogenic stimulation. It also supplied additional evidence that the interstitial cells of the testis underwent a gradual change to malignancy.

Finally, the over-all results of this study are applicable to the problem of hormone-dependent tumors in 2 respects. First, they support a recent statement of Foulds (*25*) that responsiveness of tumors "is not an all-or-

none phenomenon" and suggest that, in the spectrum of hormone-dependent tumors, induced testicular tumors of the mouse occupy a position of limited dependence from which they can progress to independence upon removal of the hormone or during serial passage in estrogenized hosts. Second, the use of stilbestrol-cholesterol pellets gives the investigator some control over the production of hormone-dependent tumors or independent tumors, which are histologically indistinguishable. It also enables him to study the sequence of events in testes containing tumors growing progressively, receding, or dormant.

Summary

Stilbestrol-cholesterol pellets containing 20 percent stilbestrol were inserted subcutaneously into 2-month-old strain BALB/c male mice to study the effects of discontinuation of estrogenic stimulation upon (1) established testicular tumors and (2) the development of tumors in testes of mice exposed to the hormone for limited periods of time.

The estrogen induced a high incidence of interstitial-cell tumors of the testis, many of which were bilateral. When pellets were removed from mice with testes measuring 10 to 12 mm. in length, about half regressed to normal size while the remainder continued to enlarge. When mice whose testes had receded to normal received a second pellet, their tumors recurred promptly. In some mice tumors recurred spontaneously.

When mice that had their first pellets removed 5 to 9 months earlier in the absence of any testicular enlargement received second pellets, 50 percent developed testicular tumors within 4 months. Controls receiving their first pellets showed a tumor incidence of only 7 percent within 4 months.

Mice that had their pellets excised 10 to 16 months earlier in the absence of testicular enlargement were killed and their testes examined microscopically. Areas of interstitial-cell hyperplasia or changes were found in many of their testes. Testicular tumors arose in some mice from 1 to 12 months after removal of pellets in the absence of testicular enlargement.

These observations indicate the long persistence of interstitial-cell changes initiated by stilbestrol and suggest that the changes were irreversible but not sufficient, in most instances, for further progression unless the mice received a second hormonal stimulus.

Transplantation of testicular tumors that arose in the presence or absence of stilbestrol pellets or those that continued to grow upon removal of pellets resulted in the production of many hormone-independent tumors.

These findings suggest that induced testicular tumors of the mouse have limited dependence upon hormonal stimulation for progression and can achieve independence when the estrogen is removed from hosts carrying primary tumors or in serial passages through estrogenized hosts.

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Testicular tumors and other effects of 20 percent diethylstilbestrol-cholesterol pellets (weight approximately 5 mg.) implanted subcutaneously in strain BALB/c male mice at 2 months of age. All stained with hematoxylin and eosin.

PLATE 1

FIGURE 1.—Essentially normal testes in mouse that carried a pellet for 28 weeks and was killed at 19.5 months of age, or 10 months after removal of pellet. × 5.5

FIGURE 2.—Bilateral solid interstitial-cell tumors of the testes in mouse, which appeared 5 months after pellet implantation; animal killed at 10.5 months of age, or 8.5 months after pellet implantation. × 5.5

1

2

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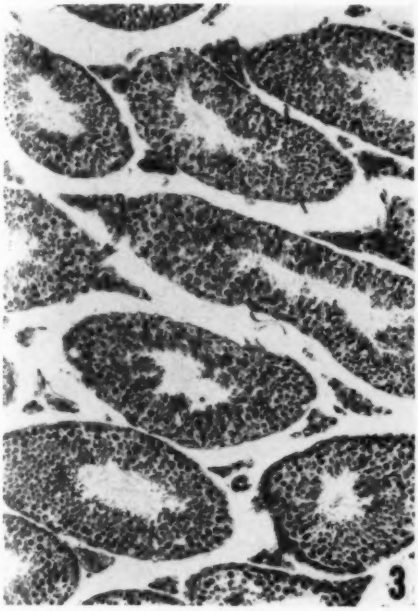
PLATE 2

FIGURE 3.—Essentially normal testis from mouse of figure 1. *Note* sparse interstitial-cell elements. Some atrophy of spermatogenic elements, not unusual for a 19-month-old animal. × 100

FIGURE 4.—Same as figure 3. × 400

FIGURE 5.—Interstitial-cell changes in mouse implanted with pellet for 16 weeks and killed at 19 months of age, or 13 months after removal of pellet. Showing persistence of interstitial-cell hyperplasia and presence of large brown cells. × 100

FIGURE 6.—Same as figure 5. × 400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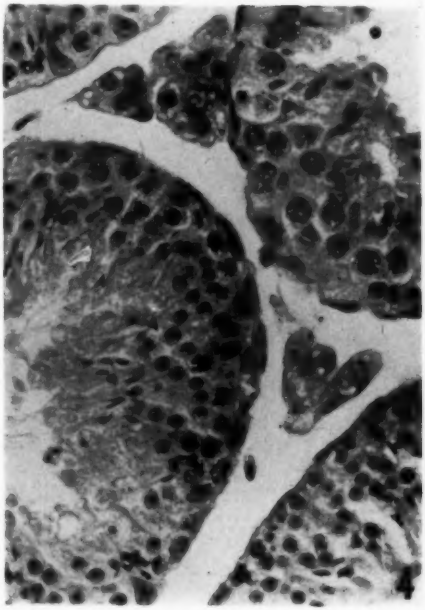
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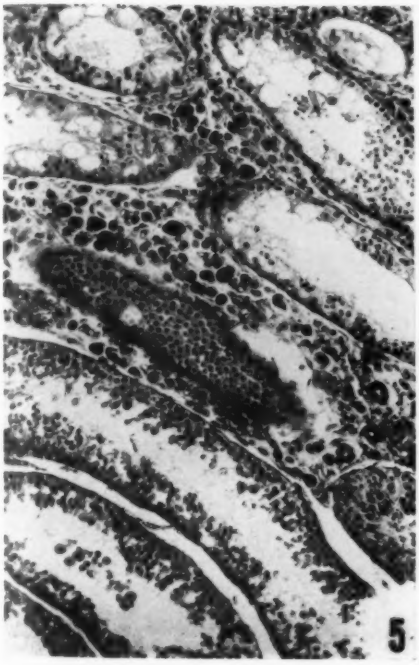
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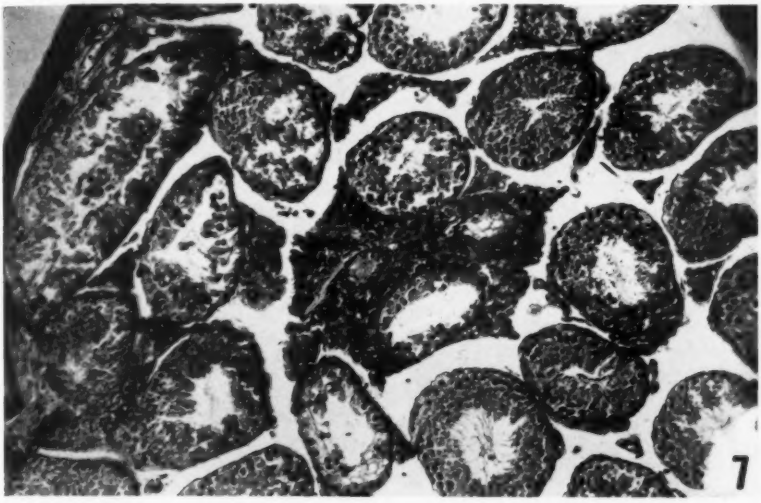
PLATE 3

FIGURE 7.—Small hyperplastic area of interstitial cells in mouse implanted with pellet for 16 weeks and killed at 22 months of age, or 15 months after removal of pellet. Showing persistence but apparent lack of continued growth. × 100

FIGURE 8.—Same as figure 7. × 500



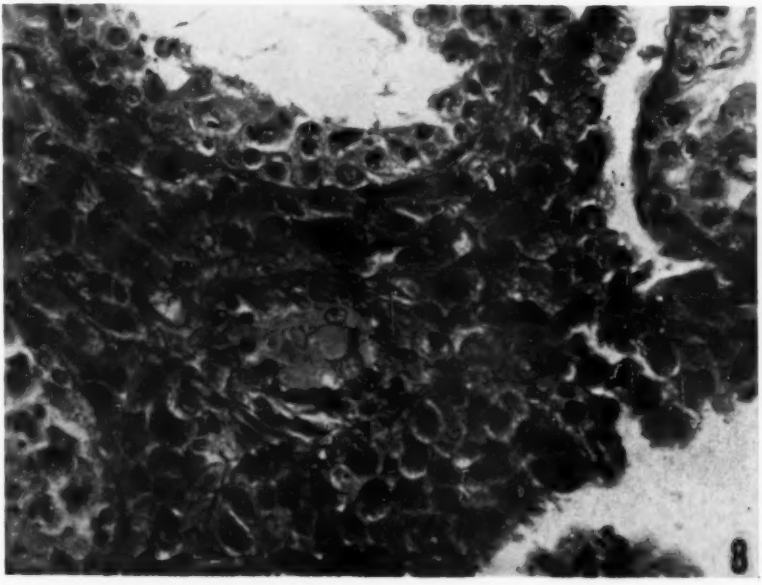


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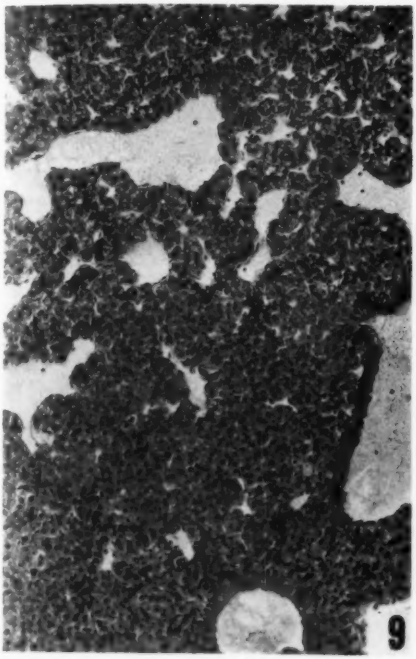
PLATE 4

FIGURE 9.—Interstitial-cell tumor of the testis, 8.5 months after implantation of pellet. Showing the adenomatous pattern of large, uniform cells around capillary spaces lined with endothelium. × 100

FIGURE 10.—Same as figure 9. × 400

FIGURE 11.—Interstitial-cell tumor of the testis, 8.5 months after implantation of pellet. Showing zone of large, clear cells ("second generation cells" of Hooker and Pfeiffer) and zone of smaller, more fusiform cells ("third generation cells" of Hooker and Pfeiffer). × 100

FIGURE 12.—Same as figure 11. × 400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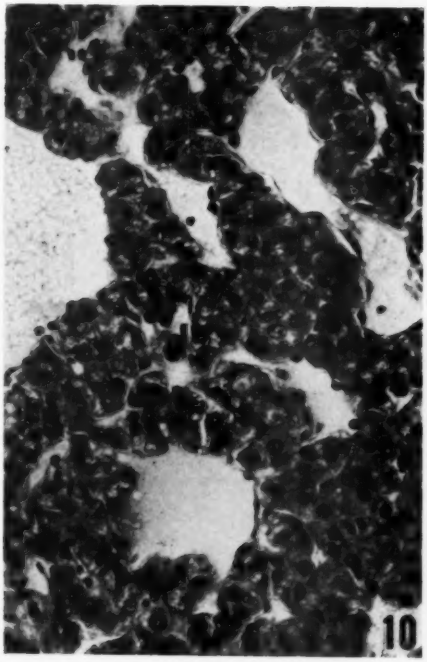
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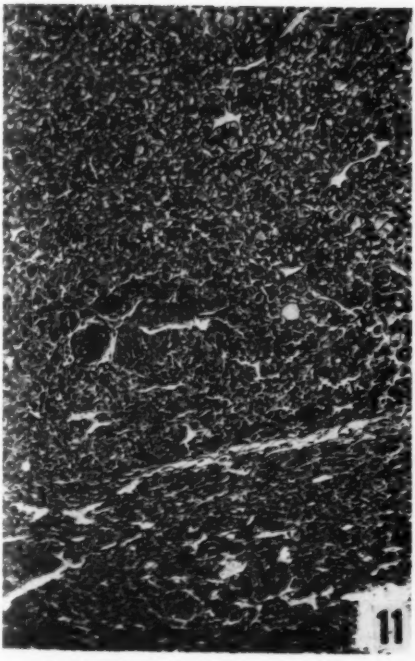
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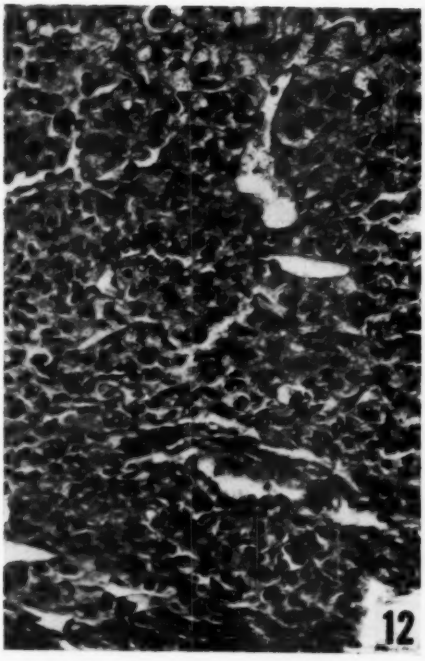
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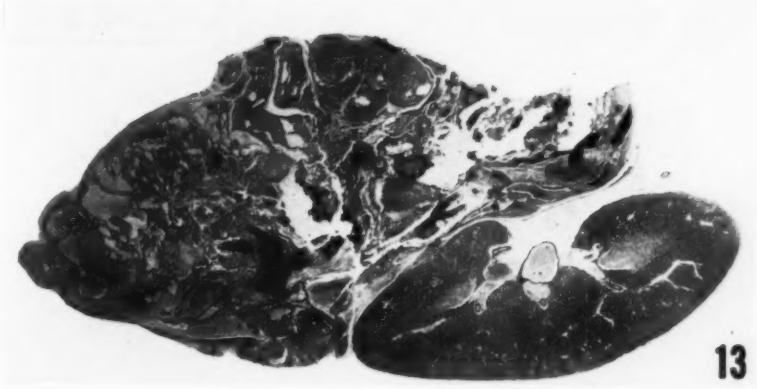
PLATE 5

FIGURE 13.—Metastasis of testicular tumor to the left epirenal area in mouse 12.5 months old, or 10.5 months after implantation of stilbestrol pellet. Animal developed testicular tumor 4.5 months after pellet implant. *Note* adrenal on the *left*, with large tumor mass and hemorrhage between kidney and adrenal. ×6

FIGURE 14.—Detail of mass, showing presence of large, uniform oval cells and more fusiform elements in the metastasis. × 100

FIGURE 15.—Metastases of testicular tumor in omentum, in a mouse with bilateral testicular tumors, 11 months after implantation of stilbestrol pellet. × 100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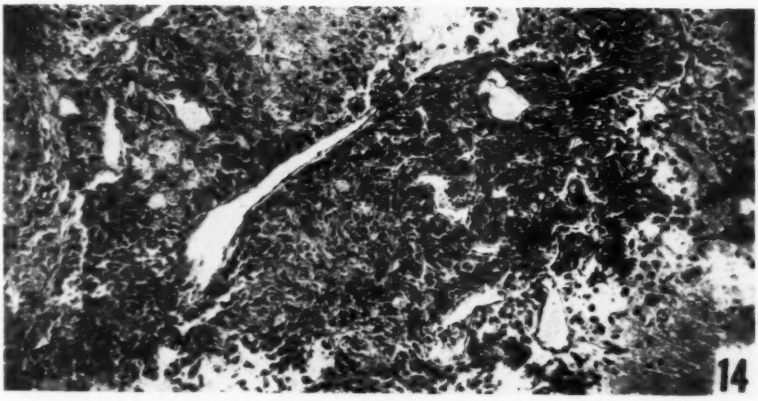
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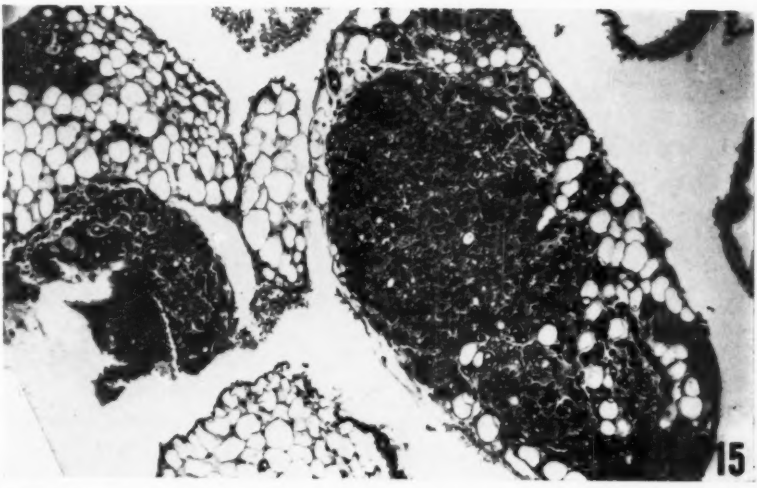
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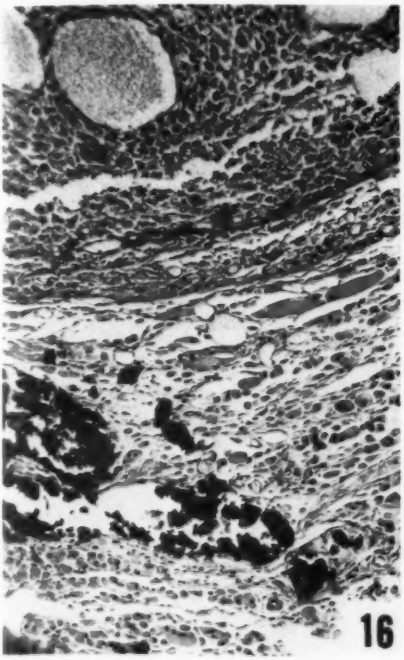
PLATE 6

FIGURE 16.—Atrophy of spermatogenic elements, degenerative changes, and persistence of tumor nodule in mouse implanted with pellet for 24 weeks and killed at 20.5 months of age, or 12 months after removal of pellet. × 100

FIGURE 17.—Atrophy, degenerative changes, and calcification and persistence of tumor in right testis of mouse that developed gross bilateral testicular tumors 7.5 months after pellet implantation, at which time the pellet was removed and testes returned to normal size. Animal killed 9 months later. × 100

FIGURE 18.—Area of almost complete atrophy and diffuse calcification in left testis of same mouse. × 400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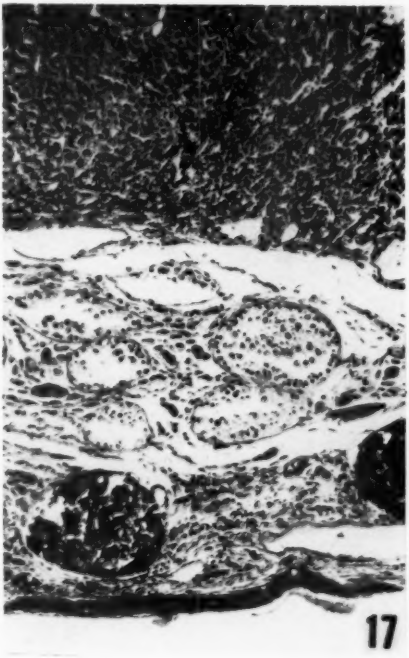
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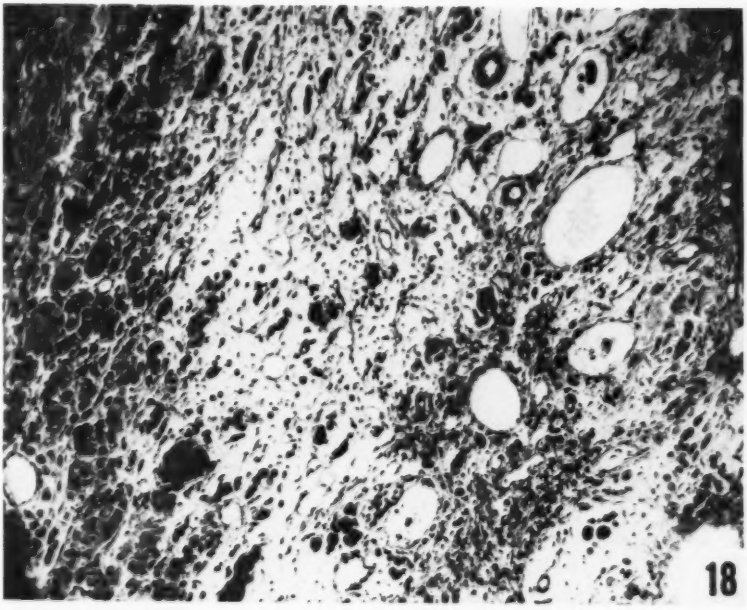
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PLATE 7

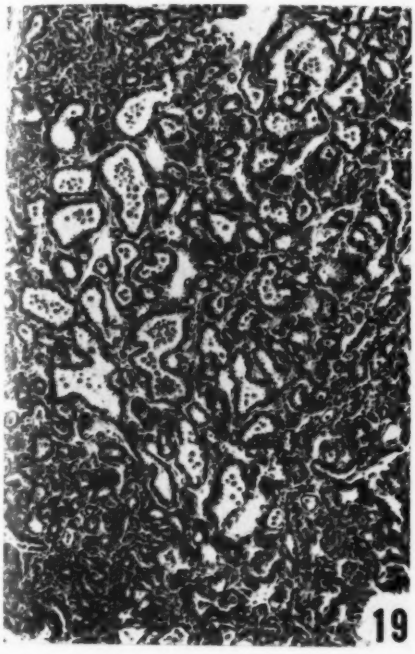
Metastasis of primary pulmonary tumor to atrophic testicular tumor in BALB/c mouse, 19 months old, implanted with stilbestrol pellet when 2 months of age. Gross testicular tumor was present at 6.5 months, the testis returning to normal size following removal of pellet.

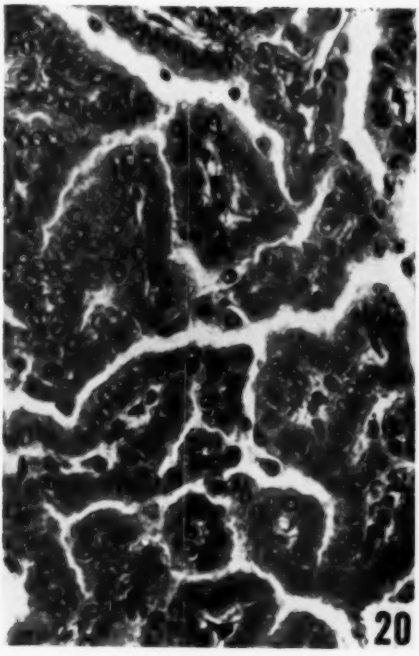
FIGURE 19.—Primary pulmonary tumor. × 100

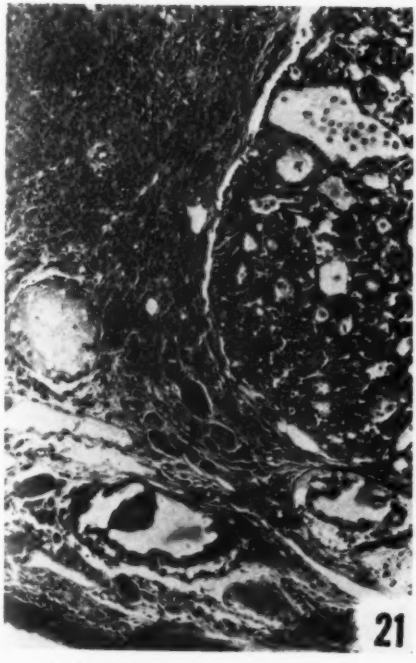
FIGURE 20.—Same as figure 19. × 400

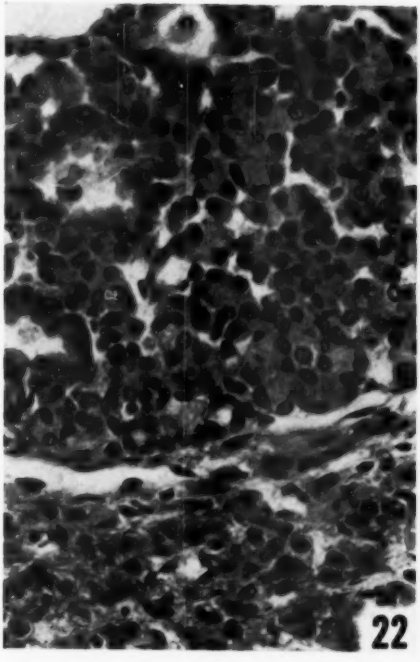
FIGURE 21.—Testis, showing atrophy and calcification, with interstitial-cell tumor on *left* and pulmonary tumor metastasis on *right*. × 100

FIGURE 22.—Same as figure 21. Showing zone between interstitial-cell tumor cells (*lower*) and pulmonary tumor cells (*upper*). × 400









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Experimental Carcinogenicity of Coal-Tar Fractions: The Carcinogenicity of Creosote Oils [1,2,3]

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Coal gases, light oils, and tars are evolved as the volatile moieties during the coal-coking process. Of these distillates, coal tar has been recognized as a carcinogenic substance for humans at least since 1875, when von Volkman reported on the prevalence of skin cancer among the distillers of lignite tars in Saxony (*1*). By 1924, enough epidemiologic evidence had been accumulated to justify a report that high-temperature tar, together with its pitch, produced far more cases of industrial cancer than all other materials put together (*2*). Experimental studies supported the early epidemiologic findings. The classical experiments of Yamagiwa and Itchikawa in 1914 produced the first experimentally induced skin tumors by utilizing a high-temperature coal tar applied to the skin of rabbits (*3*), while the first experimental tumors in mice were induced in similar fashion by Tsutsui in 1918 (*4*). Later studies, notably those by Deelman (*5*), supported the epidemiologic evidence that both high- and low-temperature tars were carcinogenic, and that the high-temperature distillate was the more potent of the 2. Concurrent demonstrations of experimental carcinogenicity with pitch (*6*) and the isolation of benzo[*a*]pyrene from a high-temperature tar and a pitch oil (*7,8*) since then have not only established those industrial products as carcinogens for man and the mouse but also have identified at least 1 of the potent agents responsible for their activity.

The crude industrial fractions distilled from high-temperature coal tars are designated light oil, middle oil or naphthalene crude cut, heavy creosote oil, anthracene oil, and pitch (table 1) (*9*). A clear-cut separation of tar into these distinctive fractions is usually not practiced on an industrial scale. In fact, such terms as creosote, heavy oil, and anthracene oil have been used synonymously, while certain industries to this day designate

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[2] The work reported here is supported by a grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[3] This paper was delivered in part at the meetings of the American Association for Cancer Research, San Francisco, April, 1955, and at the meetings of the American Industrial Hygiene Association, Philadelphia, April, 1956.

[4] With the technical assistance of Hazel Li, Raymond Wright, Dolores Stanton, Ross Walker, and John E. Thomas.

some pitches as "tars." Most of the marketed oil products are blends or smears, containing both creosote and anthracene oils plus varying amounts of the residual fractions from the middle-oil cut—the fraction shown in table 1 to have a boiling range of 200 to 250° C.

TABLE 1.—*Crude coal-tar distillates*

Industrial cut	Approximate distillation range (° C.)	Fraction recovered from tar (*percent*)	Principal components of fraction
Light oil	200	5	Benzene, toluene, xylene, solvent naphtha.
Middle oil	200–250	17	Tar acids, tar bases, naphthalene, light creosote oil (residue).
Creosote (heavy) oil	250–300	7	Methylated naphthalenes, acenaphthene.
Anthracene oil	300–350	9	Fluorene, phenanthrene, anthracene, carbazole.
Pitch	>350	62	Pitch oil, carbon.

This lack of specificity in terminology, coupled with the fact that industrial exposures may involve any or all of these fractions as well as exposure to whole tar during the worker's period of employment, have complicated the epidemiologic evidence on the tumor-provoking activity of individual fractional tar distillates. Yet, an impressive body of industrial data has been compiled that shows that a cancer-producing substance for man is present, at least in the higher-boiling tar fractions; namely, in the heavy creosote oil, in anthracene oil, and in pitch (*2,10,11*). Experimental evidence in the past has supplemented this only in part, by suggesting that the carcinogen is closely associated with benzo[*a*]pyrene (*2,12*).

Apparently no conclusive experimental evidence on the carcinogenicity of creosote oils *per se* was reported prior to the era of chemical carcinogenesis (*2,6,13*), while the lack of pertinent reports in the literature since that time probably reflects an attitude stated editorially in 1931, ". . . that painting with crude tar should henceforward be considered obsolete for any purpose where the actual process of carcinogenesis is under study. All such experiments should now be carried out with pure substances . . . ." (*13,14*). This attitude still prevails today despite the fact that the actual process of environmental carcinogenesis usually involves crude mixtures, recognition of which is indispensable for environmental-cancer prevention.

The industrial indications of a carcinogenic hazard in creosote oils, and the need for further experimental evidence on this score, prompted the study which is now reported in part. This study seeks to determine experimentally the relative potencies of industrial tar distillates, to estimate comparative carcinogenic activity in terms of the activity of known amounts of benzo[*a*]pyrene, and to analyze the active tar fractions for their benzopyrene content.

Material and Methods

The biologic effects of only 2 creosote oils are presented at this time. Both are fractions from a high-temperature horizontal by-product—coke-oven tar. One is a crystal-free oil, marketed as a blend of creosote, anthracene oils, and the oil drained from the naphthalene recovery operation. This blend of oils has been described as nontoxic to humans and is used principally for the preservation of wood (*9*). The second sample, considered a light creosote oil industrially (table 1), is the residual oil drained from the naphthalene recovery operation.

For biologic tests, the blended oil was used at 2 dilution levels, 20 and 80 percent, in toluene. The light creosote was tested only at a 50 percent concentration. In estimating comparative carcinogenicity, "standard" carcinogenic solutions were 0.05 and 0.25 percent benzopyrene in toluene (weight per volume).[5] All solutions were applied dropwise to the shaved interscapular skin of C57L male or female mice, as shown in the tables. Exposures consisted of 1 drop of solution applied 3 times a week with calibrated, blunted 20-gauge needle droppers that delivered approximately 0.008 cc. toluene per drop. Applications were repeated for the life span of the animal or until a persistent papilloma developed at the site of application. Mice dying before the induction of the first papilloma in a group were discarded. All mice surviving to that time and beyond were considered to have been "effectively exposed."

Results

A. Blended creosote.—Tables 2 and 3 present a comparison of the relative carcinogenic potencies of the 20 and 80 percent dilutions of the blended creosote oil in toluene with 0.05 and 0.25 percent benzopyrene in toluene. In the case of the weaker positive control—0.05 percent benzopyrene solution—8 out of 9 mice effectively exposed developed papillomas, whereas in each of the other 3 groups, 8 out of 8 developed the lesions. The papilloma induction time for the lower-concentration *control* group (0.05% benzopyrene) ranged from 22 to 58 weeks, whereas the induction time for the lower-concentration *test* group (20% blended creosote) ranged from 22 to 43 weeks. The papilloma induction time for the higher-concentration *control* group (0.25% benzopyrene) ranged from 14 to 25 weeks, while in the higher-concentration *test* group (80% blended creosote) the time ranged from 19 to 34 weeks. The inverse relationship between dose or carcinogenic potency and papilloma induction time has been reported previously for benzopyrene (*15*). These results indicate that the relationship holds as well for crude mixtures as for a chemically purified agent.

The types of tumors induced by the above treatments are presented in table 4. Papillomas developed on all animals that received repeated

[5] Tentative biologic standards for carcinogenic potency in our laboratory are based on skin-tumor induction with graded doses of benzo[a]pyrene, a chemical carcinogen originally isolated as a trace component of a high-temperature coal tar (*7*). All other factors being equivalent, the potency of this chemical carcinogen has been found to be a function of its dosage in the biologic test (*15*).

TABLE 2.—*Comparative carcinogenicity of a blended creosote oil and benzo[a]pyrene on C57L female mice, 10–12 weeks old on initial exposure*

Test material*	Concentration in toluene, (*percent*)	Number mice with tumors / Number mice effectively exposed	Number mice with tumors in relation to time of appearance (*weeks*)												
			14	18–20	21–23	24–26	27–29	30–32	33–35	36–38	39–41	42–44	45–47	48–50	58
Benzo[a]pyrene.....	0. 25	8/8	2	3	2	1									
Blended creosote.....	80	8/8		1	4	1	1		1						
Blended creosote.....	20	8/8			2	2	1	1	1			1			
Benzo[a]pyrene.....	0. 05	8/9			1			1	1	1		2	1		1
Toluene (control).....	00. 00	0/10													

*Applied dropwise with a blunted 20-gauge needle, 1 drop to interscapular skin 3 times per week (0.0087–0.009 cc. per drop).

TABLE 3.—*Comparative carcinogenicity of a blended creosote oil and of benzo[a]pyrene of C57L female mice, 10–12 weeks old on initial exposure*

Test material*	Concentration in toluene (*percent*)	Time of papilloma appearance† (*weeks*)
Benzo[*a*]pyrene	0. 25	18
Blended creosote	80	21
Blended creosote	20	26
Benzo[*a*]pyrene	0. 05	43
Toluene (control)	—	‡

*Applied dropwise with a blunted 20-gauge needle, 1 drop to interscapular skin 3 times per week (0.0087-0.009 cc. per drop).

†In 50 percent of the animals.

‡No epidermoid tumors during life span.

applications of 0.25 percent benzopyrene in toluene, and epidermoid carcinomas developed subsequently at all sites of exposure. In the lungs or regional lymph nodes, 2 of the 8 cancerous mice showed grossly demonstrable metastases, which were confirmed histologically. All mice exposed to both dilutions of the creosote oil also developed papillomas. Seven of the 8 exposed to each concentration progressed to malignancy. One tumor regressed before the death of a mouse in the 80 percent creosote group, while 1 papilloma on a mouse in the 20 percent group remained as a grossly obvious but nonprogressive wart until the mouse died. Among the creosote-treated animals that developed carcinomas at the site of exposure, it is noted that a substantial fraction had metastatic growths. (Table 4 and figs. 1–4.)

In view of the slightly higher incidence of tumors among the 20 percent creosote-treated mice, the shorter period of chronic exposure for tumor development, and the slightly greater extent of metastasis, it appears that the potency of the creosote at the 20 percent dilution level exceeds the activity of the 0.05 percent benzopyrene standard. Yet, despite more rapid papilloma induction in all creosote-treated animals, the failure of 2 of these mice to develop carcinomas suggests the possibility that the biologic effects of benzopyrene and of the creosote oil may not be quantitatively identical, even though both produce the same qualitative end result—epidermoid carcinomas that metastasize.

It was thought that benzopyrene, if present in this blend, would probably be concentrated in the anthracene-oil component. Consequently, a sample of unblended anthracene oil or Base Stream oil was fractionated by elution chromatography. The fractions were collected and analyzed for their benzopyrene content in an affiliated laboratory with a Cary recording ultraviolet spectrophotometer.[6] These analyses indicated no detectable benzo[*a*]pyrene in the original sample of anthracene oil. The limit of detectability was 0.03 percent benzopyrene. In view of a carcinogenic potency for the anthracene oil, approximating that of 0.25

[6] We are indebted to C. B. Willingham and L. Sullivan, Department of Research in Physical Chemistry, Mellon Institute, Pittsburgh, Pa., for analysis of these materials.

TABLE 4.—*Comparative carcinogenicity of a blended creosote oil and of benzo[a]pyrene* on C57L female mice, 10–12 weeks old on initial exposure*

Test material	Concentration in toluene (*percent*)	Number mice with tumors / Number mice effectively exposed	Tumor static (S) or regressed (R)	Epidermoid cancer of exposed skin	Number with metastasis / Number with epidermoid cancer	Lung metastasis	Regional lymph node metastasis
Benzo[*a*]pyrene	0. 25	8/8	—	8	2/8	2	1
Blended creosote	80	8/8	1 (R)	7	3/7	1	3
Blended creosote	20	8/8	1 (S)	7	2/7	1	2
Benzo[*a*]pyrene	0. 05	8/9	1 (R)	7	1/7	1	1

*Applied dropwise to interscapular skin with a blunted 20-gauge needle, 1 drop 3 times per week (0.0087–0.009 cc. per drop).

TABLE 5.—*Comparative carcinogenicity of a light creosote oil and of benzo[a]pyrene and their combined effects on C57L male mice, 8–11 weeks old on initial exposure*

Test material applied to skin 3 times per week	Number mice with tumors / Number mice surviving 20 or more weeks' treatment	Number tumors in relation to time of appearance (*weeks*)					
		6–20	21–25	26–30	31–35	36–40	41–45
1 drop 0.05% benzo[a]pyrene in toluene + 1 drop toluene	9/10*		1	2	3		3
1 drop 50% light creosote oil in toluene + 1 drop toluene	11/11		3		2	1	5
1 drop 50% light creosote oil in toluene + 1 drop 0.05% benzo[a]pyrene in toluene	10/10	2	3		4	1	

*After 55 weeks' exposure, 1 mouse died with a hepatocellular carcinoma but no skin tumor.

percent benzopyrene, it is apparent that the activity of this creosote cannot be due to its benzopyrene content, if indeed any benzopyrene is present at all.

B. Light creosote oil.—Table 5 shows comparative biologic effects of benzopyrene and of light creosote, the residual oil drained from the naphthalene stills and used to dilute heavy creosote. Following repeated applications of the "standard" carcinogen, 0.05 percent benzopyrene in toluene, skin tumors on 9 of 10 mice were recorded after exposures ranging from 25 to 44 weeks. In comparison, the 50 percent solution of light creosote oil in toluene produced tumors on all mice in this group after periods that ranged from 22 to 41 weeks. The capacity of benzopyrene and light creosote oil together to summate their individual biologic effects was also demonstrated, as shown in table 5. Tumor induction time was obviously reduced for the mice receiving both agents, as compared with tumor induction time for either agent alone.

Chromatographic fractionation and ultraviolet analysis of this light creosote oil also gave no evidence of the presence of benzopyrene in any of its fractions.[7] The limit of detectability for this sample as a whole was 0.005 percent benzopyrene, an amount roughly one tenth that required to duplicate the biologic activity of the diluted agent.

Discussion

These experiments clearly indicate that creosote oils from high-temperature coal tars are carcinogenic and that their activity is due to agents

[7] We are indebted to C. B. Willingham and L. Sullivan, Department of Research in Physical Chemistry, Mellon Institute, Pittsburgh, Pa., for analysis of these materials.

other than benzopyrene that may be distilled from tar in the temperature range 200 to 250° C. as well as at higher temperatures. Previous reports on this subject include the work of Kennaway (*2*), Woglom (*6*), Henry (*10*), Poel and Kammer (*16*), and Hueper (*17*). In a paper on the cancer-producing factor in tar, Kennaway reported that industrial evidence appeared to be almost wholly negative as regards the potency of "light oil and carbolic oil" (naphthalene crude cut). Industrial evidence of heavy creosote-oil carcinogenicity was cited, however. As regards tumor induction in the laboratory, he wrote, "Apparently, no conclusive experiments have been made with the creosote fraction alone."

Neither epidemiologic nor experimental data could be found to suggest a carcinogenic hazard associated with the light creosote oil. Cabot (*18*) reported his impression that the skin irritation due to handling of anthracene oil or pitch disappeared when these materials contained 10 percent or more of the lower-boiling fractions. In an experimental study with mice, he reported that the entire creosote-oil fraction and the neutral residue retarded tumor production with benzo[*a*]pyrene. A subsequent study by Sall and Shear (*19*) considered the possibility that other constituents of coal tar besides benzopyrene play a part in the carcinogenic action of the crude tar. The phenolic fraction of their creosote oil appeared to retard the production of skin tumors with benzopyrene, and the basic fraction appeared to promote it, while the neutral fraction was not significantly carcinogenic. Their creosote oil and fractions were obtained from a vertical-retort coal tar, while the products tested in our laboratory were high-temperature coal-coking by-products. Variations in the composition of tars obtained by coal carbonization at different temperatures probably account for some of the differences in activity reported, although Deelman's findings (*5*) suggest the need for more experimental work with low-temperature distillates. In contrast with the findings of Sall and Shear (*19*), Woodhouse (*20*) tested a specimen of creosote oil and found it experimentally carcinogenic. Unfortunately, identification of the sample as to source, physical characteristics, nature of the parent distillate, etc., was omitted, thus making speculative any inferences or comparison of that observation with others.

In a recent demonstration that whole tar contains potent constituents apart from benzo[*a*]pyrene, Berenblum and Schoental (*12*) isolated 2 major carcinogenic fractions that were entirely free from benzo[*a*]pyrene. One of these, eluted from their chromatography column just before benzopyrene, was highly carcinogenic for rabbit skin but not for the skin of mice; the second, a fraction eluted after benzopyrene, they reported to be carcinogenic in both mice and rabbits. The activity indicated by our study probably is not due to the same agents that they concentrated, since they started with whole tar and demonstrated carcinogenic activity for fractions immediately preceding and following benzopyrene elution, while our starting materials were creosote oils distilled at temperatures below that needed to reach the benzopyrene-containing cut. Although there is a possibility that the fraction they eluted before benzopyrene

might be a component of anthracene and heavy creosote oils, it does not account for all the activity of the creosotes, in view of the potency of our samples in mouse skin and the lack of potency reported for their fractions. Apparently, then, the span of carcinogenic fractions that may be distilled from whole tar extends from the pitch residue, at least up to the light creosote oil of the naphthalene crude cut, and includes simpler or lower-boiling molecules than those associated with benzopyrene, as well as such complex ones.

An indication that the activity of creosotes is not limited merely to 1 strain has already been demonstrated by tumor induction on C57BL/6 mice (*16*). In experimental work now under way, tumor induction with these materials has also been demonstrated with A/He, DBA/2, SWR, C3H, and RAP mice. Although differences in response apparently due to sex, strain, and age have been observed, the sum of such biologic factors appears merely to condition the carcinogenic response. In no instance does it protect against tumor induction under our experimental conditions, unless a decreased life span and death before tumor development are considered a form of cancer protection.

It is worth noting that experimental papillomas, induced by coal distillates or by benzopyrene, may regress. The regression of so-called "tar-warts" was first observed and reported by clinicians, among cases of people working with coal-tar by-products (*1,6*). Experimental duplication of this condition in animals has provided the investigator with a research tool not available ordinarily to the clinician and epidemiologist for investigating tumor regression. As a result of an experimental approach to this problem, it was previously suggested that chemical carcinogenesis may be a multistage process in which malignancy is developed by the tumor as a terminal phenomenon (*21*). On the basis of recent experimental findings on skin carcinogenesis with both tar distillates and pure chemical carcinogens, it appears that benign papillomas, localized malignant papillomas, and invasive epitheliomas, rather than representing qualitatively different neoplastic entities, actually represent different sequential stages in a multistage sequence of carcinogenesis. These experimental findings, therefore, re-emphasize the need for adequate prophylactic programs in industry that stress early detection of pre-malignant changes, as a step toward the elimination of industrial cancer.

Summary

1) The experimental carcinogenicity of light and heavy creosote oils from high-temperature coke-oven tars has been demonstrated by applications to the skin of mice.

2) Carcinogenic potency has been detected in light creosote oils from industrial samples distilled at temperatures as low as 200 to 250° C.

3) The apparent absence of benzo[*a*]pyrene in the creosote oils tested confirms the impression that other potent carcinogenic agents are present in coal tar.

4) The induction of tumors that subsequently regress supports the im-

pression that chemical carcinogenesis is a multistage process in which malignancy is developed in the neoplasm as a terminal manifestation.

5) The need is re-emphasized for adequate prophylactic programs in industry that stress early detection of pre-malignant changes, as a step toward the elimination of industrial cancer.

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PLATES

PLATE 8

FIGURE 1.—Keratinizing epidermoid carcinoma on a DBA/2 male mouse after thrice-weekly applications of an 80 percent creosote-oil blend in toluene for 46 weeks. One drop of approximately 0.008 cc. of creosote solution was applied to the interscapular skin in each exposure.

FIGURE 2.—Histologic details of the primary lesion on a C57L female mouse after topical applications of 50 percent light creosote oil in toluene. A squamous-cell carcinoma is seen under the markedly acanthotic and hyperkeratotic epidermis.

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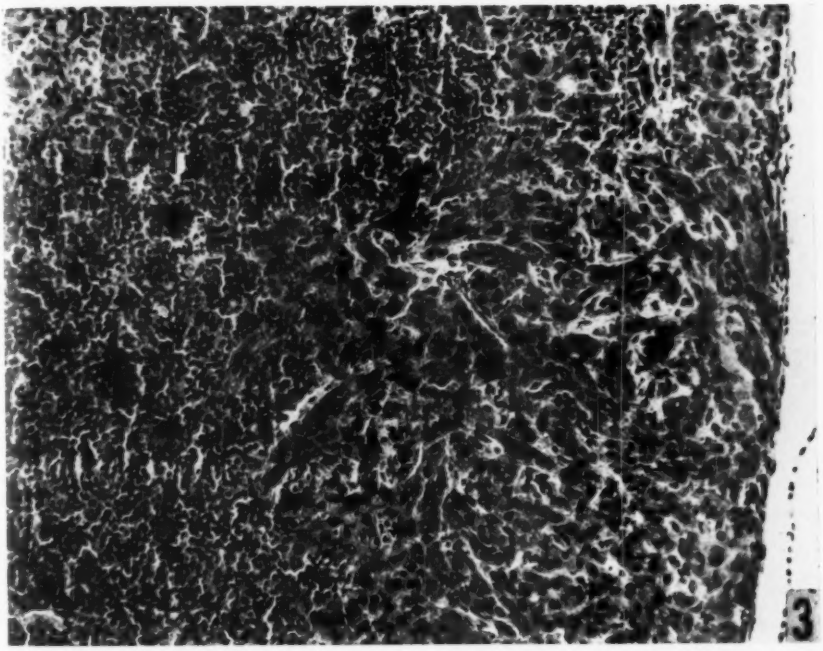
PLATE 9

FIGURE 3.—Metastases to the axillary lymph node of mouse in figure 2.

FIGURE 4.—Metastases to the lung of mouse in figure 2.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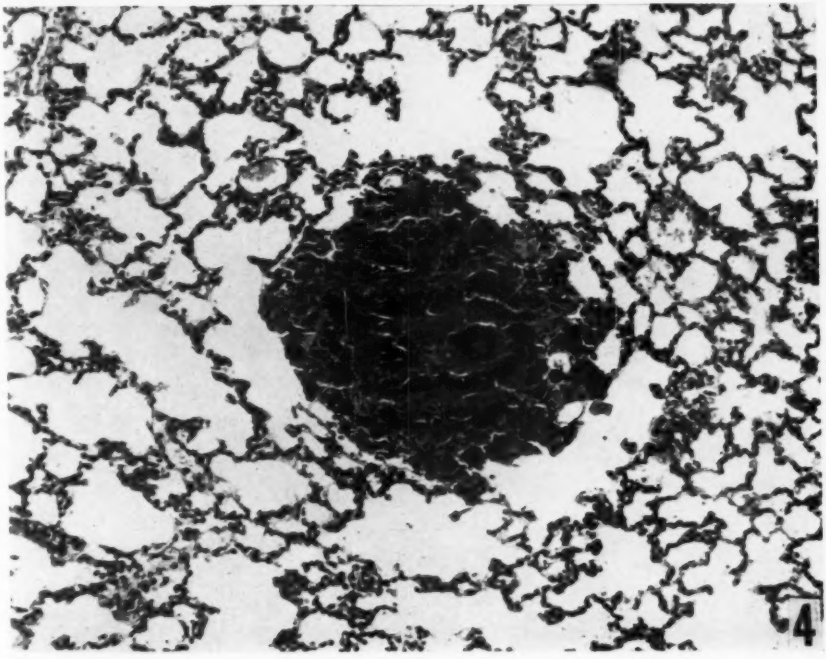
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Undulations in the Time-Response Curve for Tumor Immunity after Primary Immunization with Washed Erythrocytes [1]

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In studying the nature of tumor immunity (*1*), or immunogenetics as it is sometimes called, we have employed "incubation periods," *i.e.*, the time interval between immunizing and challenging inoculations, based upon the work of our predecessors in this field and upon well-known principles of immunology. From a practical viewpoint, this was justified by the fact that useful data resulted. However, the knowledge accumulated by many workers in this field suggested caution when transposing technical or conceptual details from other fields to this one. We therefore systematically investigated the degree of resistance in mice to tumor implantation as a function of time following a single inoculation of washed erythrocytes. The observations indicated that the curve representing the degree of response plotted against time is not like the skewed, relatively smooth time-response curve usually depicted as representing the antibody response to a single injection of antigen. Unexpectedly, we obtained a curve which seemed to reflect secondary, and probably tertiary, augmentations of the immune response. Sufficient data have been accumulated thus far to furnish a basis for useful technical details and for some interesting theoretical speculations.

Materials and Methods

Details of technique can be found in previous publications (*2,3*) and only the main features are given here.

All hosts were strain BALB/cN mice 8 to 12 weeks old. Both sexes were used, but in separate experiments. All hosts received a single immunizing injection of washed erythrocytes from strain DBA/2N adults. The antigen was administered subcutaneously in saline suspension at a dose equivalent to 0.02 cc. of blood, *i.e.*, a 0.2 cc. inoculum contained cells derived from 0.02 cc. of defibrinated blood. The tumor used for challenge was sarcoma DBA 49 (indigenous to strain DBA/2 but kills 90% of strain BALB/c mice), inoculated subcutaneously as a 50 percent suspension in saline, at a dose of 0.025 cc. Only 1 tumor inoculation was given to each host.

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In all, 7 experiments involving 1,113 hosts were performed; 5 experiments involved a total of 806 males and 2 experiments involved a total of 307 females. For each experiment a large group of mice, matched for age and sex, was given the standard dose of erythrocytes. The day of immunization was called day 0. At arbitrarily selected successive intervals thereafter, samples of the large group, usually 10 to 20 mice, were singled out and each mouse in the sample group was inoculated with a standard dose of tumor as a test of resistance on that day. The samples were chosen so as to distribute uniformly the time elapsed in preparing and administering the washed cells but were random in other respects. Subsequently, the test groups were inspected at weekly intervals for the development of a growing tumor. The data are based upon the number of tumors above a minimal size (7×7 mm.) present at 3 weeks. Long experience with several thousand similar tumors in similar hosts has shown that, for these materials, such data accurately reflect the results that would be seen by observing the animals until death.

In order to ascertain that the many tumor suspensions used during the course of this work were viable and typical of the tumor, small control groups (9–11 mice) of untreated BALB/c mice were inoculated with the same suspensions that the test groups received. A total of 67 suspensions was inoculated into a total of 664 mice.

Because of limitations of supply, animal space, and time, the intervals adopted for individual experiments varied, but they were so selected that *a*) the specific days corresponded to those in other experiments, *b*) they overlapped, in timing, one or more other experiments, *c*) they covered a critical period on the curve, and *d*) the larger number of observations was made in the interesting period between 8 and 45 days.

Results

The results of tests on the mice for resistance to tumor inoculation are shown in table 1 and text-figure 1. No significant difference related to sex of the host was seen and the sexes are given together. The order in which the separate experiments are shown is a rearrangement to facilitate visual comparison of the fluctuations in the curves; the order in which experiments were done was as given, except that #7 actually occurred between 2 and 3. The experiments extended over a period of 4 years, and no relation between season and fluctuations in the curve was detected. The tests for viability of the tumor suspensions gave the expected results; 89.2 percent of the 664 test inoculations resulted in growing tumors, and the negative results were randomly distributed among the groups.

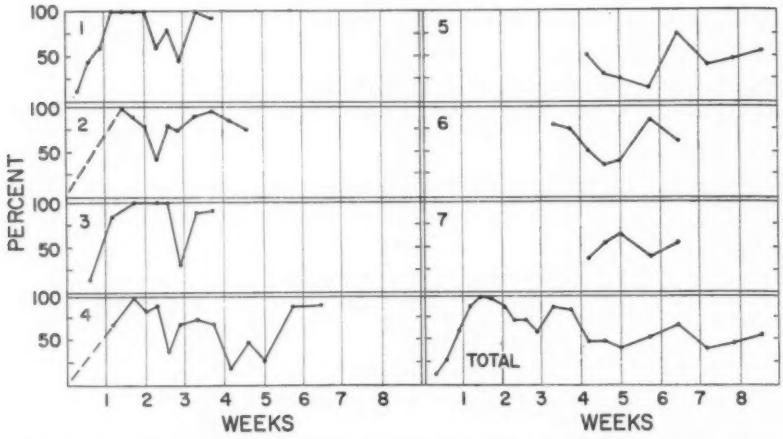
The features to be noted in the response curves are: During the 1st week there was a rise in the degree of immunity, reaching a maximum in the 2nd week. That is as expected. During the 3rd week the degree of resistance fell to a low level, followed by a return to a high level in the 4th week. In all of 4 experiments involving the 3rd week, there was a low which occurred variably from the 16th to the 20th day, and in all of 5 experiments involving the 4th week, a high level of resistance was found.

TABLE 1.—*Number of immune mice over number tested and, in parentheses, percentage of immune mice*

Day of challenge	Experiment number							Total
	1	2	3	4	5	6	7	
2	2/20 (10)							2/20 (10)
4	9/20 (45)		3/20 (15)					12/40 (30)
6	12/20 (60)							12/20 (60)
8	20/20 (100)		17/20 (85)	7/10 (70)				44/50 (88)
10	20/20 (100)	20/20 (100)						40/40 (100)
12	20/20 (100)	18/20 (90)	20/20 (100)	10/10 (100)				68/70 (97)
14	20/20 (100)	16/20 (80)		17/20 (85)				53/60 (88)
16	12/20 (60)	8/19 (42)	10/10 (100)	18/20 (90)				48/69 (70)
18	16/20 (80)	16/20 (80)	9/9 (100)	8/20 (40)				49/69 (71)
20	9/20 (45)	15/20 (75)	3/10 (30)	14/20 (70)				41/70 (59)
23	19/19 (100)	18/20 (90)	9/10 (90)	15/20 (75)		16/20 (80)		77/89 (87)
26	17/18 (94)	19/20 (95)	9/10 (90)	14/20 (70)		15/20 (75)		74/88 (84)
29		17/20 (85)		4/20 (20)	10/20 (50)	10/20 (50)	7/19 (37)	48/99 (48)
32		15/20 (75)		10/20 (50)	6/20 (30)	7/20 (35)	11/20 (55)	49/100 (49)
35				3/10 (30)	5/20 (25)	8/20 (40)	13/20 (65)	29/70 (41)
40				9/10 (90)	3/20 (15)	17/20 (85)	8/20 (40)	37/70 (53)
45				9/10 (90)	15/20 (75)	12/20 (60)	11/20 (55)	47/70 (67)
50					8/20 (40)			8/20 (40)
55					9/19 (47)			9/19 (47)
60					11/20 (55)			11/20 (55)

It is this combination of a low followed by a high that might not be expected. It may be noted that a total of 208 animals was observed during the 3rd week; of these, 138, or 66 percent, were immune, whereas 85 percent, or 151 of 177 animals, tested during the 4th week were immune. This direct comparison has a high statistical significance but, to us, the reproducibility of the phenomena lends the greater strength.

The tertiary high response is indicated by data that are not so strong as those for the secondary high response. Three experiments, #4, 5, and 6, showed a low in the 5th or 6th week followed by a high in the 6th or 7th week. This is beclouded by the results of experiment 7. In explanation of that, it may be noted that the flections of the curves for separate experiments tend to shift to slightly earlier or later positions between groups. In general, such variability between population of animals, even inbred animals, would not be surprising. It may be that in experiment 7 we



TEXT-FIGURE 1.—Percentage of mice immune to tumor at various intervals after a single inoculation of washed cells on day 0. Numerals at left of curves refer to experiment number.

accidently used a relatively small group that not only varied in time relation but also exhibited minimal fluctuations. However, one cannot tell which way to shift the curve for experiment 7, and its over-all effect is to weaken the data for a tertiary rise and to smooth out the curve for totals. Nevertheless, the tertiary rise is strongly suggested and appears in the lower curve, which represents the average response of all hosts.

Discussion

Insofar as our practical objectives are concerned, the present data seem adequate and require little discussion. Incubation periods of 8 to 12 days or of 22 to 26 days should yield consistent results in the context of our experiments, and perhaps elsewhere. Other periods may lead to confusing results.

Other aspects of these data are not so certain. Some refinements might have been obtained by extending the experiments but that would have been very expensive of time and materials. More good may come from attempts to corroborate the phenomena with other materials or under other conditions. It is probable that modifications of either would produce changes in either the timing or the shape of the curves, or both.

We have searched the literature of immunology in an attempt to find an adequate explanation for these phenomena and have found none. Some reports that are tangential to this one have been found but in them the conditions and/or the focal point were so different from ours that to cite them would be useless or unfair. In general, the greatest difference from our work in reports found was that, because of different interests, the intervals selected for observation were so spaced that fluctuations like ours would not have been seen, even if they had existed. The common statement that, after a primary inoculation the immune response usually rises rapidly, attaining a maximum in the 1st or 2nd week, followed by a

more gradual regression applies as well to our data as to any other. Had we spaced our intervals more widely we could have obtained a simple curve.

Our first interpretive thought was conditioned by the fact that, while engaged in unrelated experiments, we became suspicious that something disturbing occurred during the 3rd week under different circumstances. In that case we were working with anaphylaxis in mice (mostly strain BALB/c, but also some others). The immunizing and challenging inoculations were pooled horse serum and the test datum was anaphylactic death. The incubation period employed varied somewhat and we saw indications that the time-response curve might be depressed during the 3rd week. Tentatively, the simplest explanation for this might be that the total response was a reflection of 2 responses (to 2 different antigenic fractions) that peaked at different times. We did not investigate that further and never published it, but it suggested an interpretation of these results. An interpretation based on multiple antigens cannot be ruled out now, but it does not seem likely as an explanation of 2, or probably 3, or possibly more peaks as widely spaced in time as we found. Furthermore, in our opinion, other evidence from this laboratory, and elsewhere, suggests that it would be unwise at present to assume that the resistance to tumor implantation which is under investigation represents an undiscriminating response to any 1 or 2 of several kinds of antigen present on a single cell membrane, so that 1 peak may represent 1 antigen and a 2nd peak may represent another. The specificities usually observed in related work and the practical success of the use of an empirical incubation period in widely varied situations argue against such an interpretation—but they do not exclude it.

Our second thought, coming mainly while dealing with the earlier parts of the curve, was that the implanted cells served as an early stimulus while most of them still remained in the local depot; and at some time after 12 days, perhaps, the mass of cells broke down, flooded the system, and induced what might be called a temporary negative phase such as may follow a secondary inoculation. This idea is contrary to Murphy's report (*4*) that histologic evidence indicated few, if any, red cells remaining at the inoculation site after 8 days. Nevertheless, an attempt to get more information was made. Blood cells were tagged by administering sodium selenate containing Se^{75} to prospective strain DBA donors (unpublished experiments by the authors). The blood was harvested, washed cells were prepared, and their radioactivity was determined by means of a scintillation counter. Graded doses of cells were inoculated subcutaneously into strain BALB/c recipients. At arbitrary intervals thereafter the subcutaneous depot was excised and its activity determined and compared with the activity of a similar piece of skin from a remote site. The excess activity at the inoculation site was taken as a measure of the number of cells remaining in the subcutaneous depot. The results indicated that few if any of the cells deposited remained intact in the depot after 10 days. Such a result does not encourage one to look upon the response in the 3rd week, much less responses at later times, as a negative phase caused by

flooding the system with antigen from the inoculation site. However, that is not conclusive because we have found that washed red-cell stromata furnish an efficient antigen for tumor immunity (*3*) and it may be that stromata remain in the depot longer than does Se^{75}.

There is another aspect of this work which must be considered, even though it seems more confusing than illuminating at the moment. It is possible that our data do not represent primary responses in a strict sense although they are collected by testing animals after a single immunizing injection. In our work the test involves inoculation of a second antigen, *i.e.*, the tumor, and evaluation of the response several days later. Both the tumor and the host are living and capable of reacting in complex ways. What they show when the tumors are measured may not be a simple and direct indication of the condition of the host at the time of inoculation. This is unlike observations based on chemical equilibria between nonliving materials *in vitro*, and this distinction may separate our observations from those based upon serologic determinations of primary antibody responses. Nevertheless, the immunologic status of the host at the time of testing exerts a controlling influence upon the outcome of tumor inoculation and thus upon our observations. The fluctuations observed in the degree of response remain to be explained, regardless of whether they reflect a primary response in a strict sense or not.

It is possible that the peaks in our response curves represent differently timed maximal responses in different anatomic parts of the total responding tissues. For example, the first peak might correspond to a maximal response in the local lymphoid tissue, the second peak to a splenic response, etc. That would be in harmony with Mitchison's (*5*) observations on the time relations in passive transference of hemagglutinating activity by transplanting either lymph nodes or spleen. However, the information available to us is not sufficient to permit a conclusion in this regard.

In experiments continued along these lines we have obtained a little additional information. In a single experiment, similar observations were made on mice immunized with tumor instead of washed red cells, but no fluctuations in the response curve were observed. After the 8th day, immunity persisted at a high level for 60 days and there appeared to be little reduction at 90 days. However, no conclusion can be based on this single experiment. With regard to the participation of the spleen, a single experiment yielded information of a negative sort. Splenectomy, 7 days before the inoculation of erythrocytes, did not prevent the manifestation of a high degree of resistance 10 days after the immunizing injection. Further experiments relevant to these points are in progress.

It may be noted from the curve representing totals that there appears to be a cycle of about 16 days. We are not aware of any physiologic cycle in the mouse that has a similar periodicity and cannot suggest an explanation based upon some general physiologic characteristic of the mouse. Furthermore, one would think that with an apparently random selection of a large number of animals during a period of 4 years any normal physiologic cycle would tend to be randomized and undetectable in this work.

We do not know whether these phenomena would be seen with other materials. The only indication that we have is the suggestion, mentioned previously, from a study of anaphylactic sensitivity to horse serum in mice that something similar may occur under those conditions. More experimentation is needed. Meanwhile, it may be useful to keep these phenomena in mind when making related investigations.

Finally, we have re-examined our data from previously reported experiments in which an incubation period of 21 days was used and have found no reason to change our views. Considering only those animals in which the dose and conditions were the same, the data from (*2*) would exactly fit an interpolation from the curve given here for totals, and the data from (*3*) would very nearly fit the curve for experiment 2. But the most important point is that in those publications conclusions were based upon significant contrasts in the data from simultaneous observations.

Summary

As an adjunct to studies of induced tumor immunity in mice, experiments were designed to determine optimal intervals, following the inoculation of a small dose of washed erythrocytes, at which to test hosts for resistance to tumor inoculation. It was found that on or about the 10th day and on or about the 24th day the degree of resistance is high and they are therefore suitable incubation periods for such studies. Also found were unexpected undulations in the curve, representing the degree of resistance of the host plotted against the elapsed time after a single immunizing injection. Stated roughly, the immunity rose to 100 percent in the 2nd week, fell to 60 percent in the 3rd week, rose again to 85 percent in the 4th week, fell to 40 percent in the 5th week, and rose to 65 percent in the 6th or 7th week. Nothing like this was found in a search of the literature of immunology and no adequate explanation can be put forward with certainty at this time. Various possible influences are discussed.

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Activity of the Mammary Tumor Agent in Mice of Different Ages and Their Progeny [1, 2]

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The milk-borne (*1*) mammary tumor agent (MTA), with the properties of an infectious agent or virus, will induce mammary cancer following its administration to young, agent-free, susceptible test animals (*2–6*), but when mice older than 2 months of age are treated low incidences will usually be observed (*6–12*). However, noncancerous experimental animals, injected at any age, may become infected and transfer the agent to their progeny while nursing, and the offspring may develop mammary cancer (*3,5,8,11,12*).

An exception was noted when Dmochowski (*10*) determined that adult hybrids of the (C57BL♀ × A♂)F_1 generation would give a high incidence following repeated weekly injections of a considerable amount of dried tumor material. In adult mice of other genetic constitutions, it has been found that the incidence of mammary cancer was not related to the concentration of agent in the extracts, administered in either single or multiple doses; similar observations have been made for progeny of mothers injected at any age (*6,9,12–15*).

Since it became known that males of cancerous strains could transfer the MTA at coitus, numerous experiments have been completed on the sensitivity of females of various stocks to infection by this route (*16–28*). After females of the C (BALB/c) strain had been mated with males of the cancerous Z (C3H) stock, they showed an incidence of mammary cancer approaching 60 percent (*22,24,27,28*), whereas mammary tumors have not been recorded in those of the inbred-stock colony. Since infection with the agent usually occurred after the females of the C strain had given birth to 2 or more litters, it seemed probable that they might be satisfactory for investigating the influence of age upon the tumor-producing activity of the agent following its introduction into older mice.

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[2] Assisted by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, the Minnesota Division of the American Cancer Society, and the Graduate School Cancer Research Fund of the University of Minnesota.

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Materials and Methods

A spontaneous mammary adenocarcinoma from a female of the A stock, #9234, was transplanted into agent-free susceptible mice (Ax and ABC hybrids), and the transplanted tumors served as the source of the MTA—several tumors being used each day. The extracts, injected on May 19, 1952, were prepared from first-passage tumors, those administered the following day were from tumors of first and second transplants, while the fractions used on May 21, 22, and 23, 1952, were extracted from second-passage tumors only. The tissue was macerated in a sterile tissue press and ground with sand; distilled water was added to give a 20 percent suspension, which was then centrifuged for 5 minutes. The supernatant was recentrifuged for 20 minutes at approximately 2500 r.p.m., and the final supernatant was diluted so that the 0.5 cc. intraperitoneal injection contained the agent in either 10^{-1} or 10^{-3} gm. equivalents of tissue. With the exception of some ZBC control animals, all test animals that received a single dose (10^{-1} or 10^{-3} gm. equiv.) were treated on May 19; those which were given 5 injections (5×10^{-1}) received 1 on each day between May 19 and 23, 1952. In most instances, littermate controls were employed.

The control ZBC females were between 21 and 27 days old at the time of treatment, or the first day of multiple injections; agent-free Ax females were 102 to 141 days of age and Zb females 89 to 141 days, while the C (BALB/c) mice were of varied ages, as shown in table 2. Only noncancerous injected females that survived for more than 300 days and had at least 2 litters of progeny were tabulated. The average ages were determined from the date of birth of the animals, not from the date of introduction of MTA. All experimental females and their progeny were maintained as breeders, unless otherwise indicated. In studies where mice of different stocks were tested, the ZBC animals were always the last to be injected with any given fraction containing MTA.

Experimental Results and Discussion

When 21- to 27-day-old ZBC mice received MTA, the incidence of mammary cancer was not less than 70 percent, except in the group given 5 injections of 10^{-1} gm. equiv. of tissue, where the incidence was 59 percent (table 1). Those that were given multiple injections were litter mates of the animals that received 10^{-3} dilutions on May 19, 1952. They showed the earliest average cancer age of any group and an incidence of 74 percent. Other litter mates were used on May 23, 1952. and no variation was found between those receiving the same suspension diluted either 10- or 1,000-fold, for 90 percent had cancer at the same average cancer age. When tumors of the same passage had been assayed and differences in the activity of the agent were seen, generally, only small numbers of mice were used.

The data in table 2 concern observations for Ax, Zb, and C females, which received MTA at the ages indicated. One mammary tumor was observed among the experimental Ax females, while 3 of the 27 injected

TABLE 1.—*Development of mammary cancer in ZBC animals injected between 21 and 27 days of age with MTA obtained from transplanted tumors from the A stock*

Date of injection	Gm. equiv. injected*	Number of mice	With cancer (*percent*)	Average age (*days*)	
				Cancer developed	Died without cancer
5/19/52	10^{-3}	27	74	311	487
5/20/52	10^{-1}	10	70	415	394
5/21/52	10^{-1}	9	89	312	539
5/22/52	10^{-1}	10	70	344	581
5/23/52	10^{-1}	30	90	322	670
5/23/52	10^{-3}	29	90	325	483
5/19–5/23/52	5×10^{-1}	29	59	370	482

*Different groups received 1 injection of either 10^{-1} or 10^{-3} gm. equiv., and the last group received 5 injections of 10^{-1}—1 per day for 5 successive days.

Zb mice died with mammary cancer. Mice of both stocks that received 5×10^{-1} gm. equiv. of material (5 injections, each of 10^{-1} gm. equiv.) remained free of mammary tumors.

When the females of the C stock were recorded according to their age at the initiation of the experiment, no difference was seen between the incidence of tumors in the groups of old mice and that from the group 33 to 51 days of age. Of the C females older than 33 days of age that received the agent from either 10^{-1} or 5×10^{-1} gm. equiv. of tissue, 7 of 59, or 11.9 percent, had tumors, compared with an incidence of 41 percent found in 32 litter mates that received MTA from 10^{-3} gm. equiv. of tissue. The differences in incidence were of statistical significance (3.2 $\times$ S.E.). The C females showed entirely different results than did either the Ax or Zb animals, although in all cases MTA was from a transplanted mammary tumor from the A stock.

One C female, which received the MTA when 27 or 28 days of age, died without cancer at 764 days of age, after having 8 litters, but all others developed the disease. Again, it was evident that the cancer ages could not be correlated with the dose of MTA administered to these young C mice (table 2).

To obtain information on the development of cancer among the progeny of the injected mice, 5 females in each series were mated but some failed to have, and others failed to raise, any offspring. Consequently, the number in some groups was not adequate for reliable results (table 3). Other data showed that after Ax females had been mated with Zb males, their hybrid progeny had higher incidences of tumors than did the young obtained by crossing Ax females with males of the C stock. The hybrids with Zb maternal parents showed high incidences, regardless of the concentration of MTA administered to the Zb females or the paternal parents. At least 27 progeny were obtained in crosses between the injected C females and either Ax or Zb males, and, again, the mothers that received 5 injections, containing the more concentrated fraction of MTA, had

TABLE 2.—*Mammary-cancer data for females of C, Zb, and Ax stocks injected at different ages with extracts of transplanted mammary tumor #9234 from the A stock**

Age when injected (*days*)	1×10^{-1} gm. equiv.				5×10^{-1} gm. equiv.				1×10^{-3} gm. equiv.			
	Number of mice	With cancer (*percent*)	Average age		Number of mice	With cancer (*percent*)	Average age		Number of mice	With cancer (*percent*)	Average age	
			Cancer developed	Died without cancer			Cancer developed	Died without cancer			Cancer developed	Died without cancer
C Stock												
100–139	15	13	443	665	21	14	429	584	20	40	427	717
33–51	12	17	274	433	11	0	—	574	12	42	330	569
27–28	5	100	426	—	4	100	351	—	5	80	364	764
Zb Stock												
89–141	10	20	511	583	8	0	—	488	9	11	398	506
Ax Stock												
102–141	19	5	314	542	22	0	—	592	19	0	—	546

*These females were the mothers of the progeny recorded in table 5.

TABLE 3.—*Development of mammary cancer in progeny of C, Zb, and Ax females injected at different ages (listed in table 4) with fractions containing MTA from the A stock transplanted tumor #9234*

Parents		Age of mothers injected	Dose (*gm. equiv.*)	Progeny			
				Number of mice	With cancer (*percent*)	Average age (*days*)	
females	× males					Cancer developed	Died without cancer
Zb	Ax	135–141	5×10^{-1}	9	78	376	583
Zb	Ax	104–141	1×10^{-1}	24	100	283	—
Zb	Ax	112–141	1×10^{-2}	17	82	296	376
Ax	Zb	131–141	5×10^{-1}	10	70	370	684
Ax	Zb	134–141	1×10^{-1}	6	83	317	718
Ax	Zb	130–141	1×10^{-2}	21	57	336	571
Zb	C	89–121	5×10^{-1}	18	78	300	564
Zb	C	89–125	1×10^{-1}	17	88	293	541
Zb	C	94–118	1×10^{-2}	20	100	280	—
C	Zb	108–139	5×10^{-1}	35	6	485	638
C	Zb	108–139	1×10^{-1}	33	36	318	706
C	Zb	108–139	1×10^{-2}	33	73	299	650
Ax	C	129–131	5×10^{-1}	7	14	373	716
Ax	C	129–131	1×10^{-1}	4	75	321	705
Ax	C	130–131	1×10^{-2}	9	0	—	574
C	Ax	110–120	5×10^{-1}	27	19	506	641
C	Ax	111–120	1×10^{-1}	34	21	447	705
C	Ax	110–120	1×10^{-2}	41	46	352	693

offspring with not only the lowest incidences but also the oldest average cancer ages.

Progeny were continued from 14 females that developed mammary cancer following the administration of MTA. Data for this group are given in table 4.

Forty-four progeny were born to these cancerous mothers in their first and second litters, and 41 were born in their third and following litters. The respective cancer incidences were 55 and 56 percent, and the average ages at cancer development, 291 and 327 days; for the total number, 55 percent and 309 days. These figures may not have too much significance because the mean incidence of the progeny having C cancerous mothers that were given 5×10^{-1} gm. equiv. of tissue was 21 percent, compared with an incidence of 71 percent for those with mothers that received the extract diluted 1,000-fold. Also, all offspring born to the cancerous Zb females developed the disease, whereas the incidences for those born to C mothers varied from 0 to 100 percent, with a mean incidence of 51 percent. The 3 cancerous Zb mothers developed cancer at an average age of 473 days, 2 months later than the 11 cancerous C females, which had an average age of 430 days.

Some remarks may be in order concerning the observations made on the progeny of certain cancerous females (table 4). Female #183592 had

TABLE 4.—*Observations on progeny of cancerous females. The females were injected between 89 and 144 days of age (average of 117 days) with different amounts of MTA from A stock transplanted tumor #9234*

Females					Progeny			
Stock and number	Age at cancer development	Number of litters	Gm. equiv. injected	Mated males	Number	With cancer (*percent*)	Average (*days*)	
							Cancer developed	Died without cancer
C 183525	541	4	5×10^{-1}	Ax	3	0	—	843
C 183587	390	6	5×10^{-1}	Ax	6	50	411	568
C 183592	344	9	5×10^{-1}	Zb	7	14	685	696
C 183274	446	6	10^{-1}	Zb	4	75	232	910
C 183580	439	4	10^{-1}	Zb	5	0	—	651
C 183422	311	4	10^{-2}	Ax	6	83	249	829
C 183528	574	7	10^{-3}	Ax	7	71	378	677
C 183589	488	4	10^{-3}	Ax	14	7	429	665
C 183590	488	4	10^{-3}	Zb	3	100	303	—
C 183594	293	3	10^{-3}	Zb	3	100	321	—
C 183273	417	4	10^{-3}	Zb	11	64	296	597
Zb 175237	492	5	10^{-1}	Ax	8	100	283	—
Zb 175316	529	5	10^{-1}	Ax	3	100	298	—
Zb 175401	398	4	10^{-3}	C	5	100	246	—

mammary cancer when she was 344 days of age. Although she had nine litters, only 7 of her progeny were continued from her first, sixth, and seventh litters. All died noncancerous except 1 of the seventh litter. This C mother had received the agent administered in 5 injections, each containing 10^{-1} gm. equiv. of tissue. Another cancerous C female, #183580, had 5 progeny observed from her first and fourth litters; all died noncancerous. Following the administration of the agent at 1,000-fold dilutions, a C female had cancer when she was 488 days of age. Fourteen of her hybrid progeny were observed but only 1 had cancer. Ten of these noncancerous progeny were representatives of her third and fourth litters, and they had an average of 5.2 litters.

Fifteen additional C females, members of 3 litters, were injected with extracts of transplanted tumors when they were 27 to 28 days of age. In the group receiving 5×10^{-1} gm. equiv. of tissue, 1 female died before reaching the cancer age, another (10^{-3} gm. equiv.) died noncancerous at 764 days after having 8 litters, and the others developed mammary cancer (table 2). These experimental females were mated with C males and observations were made on their progeny and, in some instances, on descendants of succeeding generations. Irrespective of the amount of agent injected, the C females that developed mammary tumors before they were 350 days of age had an average of 4.8 litters, while those that survived longer than 400 days before they became cancerous cast an average of 8.7 litters.

The 11 offspring born to the noncancerous C female also remained free of mammary cancer, and so it must be assumed that the mother did not

become infected with the agent; all other females died with cancer, as did a majority of their progeny.

Details are given in table 5 for the progeny of the cancerous C females. The observations would not support the theory, suggested previously (*9,29,30*), that the development of mammary cancer must be influenced by the litter in which the mice were born or by the concentration of MTA. Also, in tests where MTA could not be demonstrated by bioassay of spontaneous mammary tumors (*16–18,20,25,26,28,31–33*), these data do not support the interpretation that the amount of agent might be sufficient to induce cancers in the hosts but not adequate to be biologically active in extracts of such cancers.

It may be of interest to consider selected observations concerning the development of cancer in some progeny and descendants of the cancerous C females.

Littermate females, #184706 and #184707, each received MTA from 5×10^{-1} gm. equiv. of tissue and developed mammary tumors at the ages of 313 and 430 days of age, respectively. The 6 progeny of #184706 died cancerous at an average age of 339 days, as did 71 percent of the 24 descendants of the following generation. Four of the 7 offspring of female #184707 had cancer, with an average cancer age of 272 days, while the noncancerous mice lived to an average of 721 days. Two members of the fifth litter and 21 of 24 of their progeny remained free of mammary cancer. Another litter mate of the above, #184709, developed mammary cancer at 274 days of age, following the administration of the agent diluted 1,000-fold; tumors appeared in all of her 6 progeny and 70 percent of the mice of the next generation at the respective ages of 362 and 326 days.

The MTA from 10^{-3} gm. equiv. of tissue was injected into C female #184703, and she developed cancer when 429 days of age. This female had 7 litters, and of the 7 progeny saved from the first and second litters mammary cancer arose in 6 at an average age of 312 days, compared with 324 days for 7 cancerous mice of the 8 offspring continued from subsequent litters. A spontaneous mammary tumor from a female born in the third litter to #184703 was tested for the agent by biological assay. Extracts containing either 2×10^{-2} or 10^{-3} gm. equiv. of tissue were administered to a total of 47 ZBC test animals, 24 to 26 days of age, and tumors appeared in 60 and 74 percent of the mice at average ages of 377 and 410 days, respectively.

Three of the 4 females of the second litter cast by #184703 had cancer at an average age of 330 days. Twenty-five offspring of the cancerous females were used as breeders and 11 were kept as nonbreeders; the respective incidences found in these offspring were 72 and 91 percent at average ages of 328 and 372 days. The fourth female of the second litter died without cancer when she was 709 days, after bearing 11 litters. Sixteen of the progeny of this female, #187320, had an average of 4.7 litters each, yet only 2 had mammary cancer at an average age of 708 days. These 2 cancerous females were from the first litter born to

TABLE 5.—*Observations on development of mammary cancer in progeny of cancerous C females injected with extracts containing MTA at 27 or 28 days of age*

Gm. equiv. injected into mothers	Litters 1 and 2			Litters 3 and following			All progeny		Average age (*days*)	
	Number of mice	With cancer (*percent*)	Cancer age	Number of mice	With cancer (*percent*)	Cancer age	Number of mice	With cancer (*percent*)	Cancer developed	Died without cancer
5×10^{-1}	10	70	334	17	82	284	27	78	301	541
1×10^{-1}	18	61	364	44	82	342	62	76	347	553
1×10^{-2}	13	77	304	26	77	330	39	77	321	535

#187320; 21 of their progeny and 12 from a noncancerous litter mate were maintained as breeders, and mammary cancer was found in 2—an incidence of 6 percent. While these tumors were not tested for the agent, the incidence of 6 percent was significantly different from that found among the progeny of the cancerous females of the same litter, namely, more than 70 percent.

The development of mammary cancer in mice has been shown to be dependent generally upon the interaction of 3 factors: genetic constitution, hormonal stimulation, and the mammary tumor agent (*30*, reviewed in *2*). Since this theory was advanced, it has been determined that the incidence may be similar in certain groups of breeding females in which MTA usually cannot be demonstrated by biological assay of the tumors (*16–18,20,28,31–33*), and in other susceptible breeding females possessing the agent (*34–37*), because of an inherited hormonal pattern.

After it had been shown that the administration of the agent to adult mice did not produce a high incidence of mammary cancer, it was suggested that, if the architecture of the mammary glands in mice was influenced by the presence or absence of the agent, as described by van Gulik and Korteweg (*38*) and others (*39,40*), perhaps it was necessary for the glands to develop in young mice possessing the agent in order for them to become cancerous when the animals attained the older age.

In discussing these observations, Gardner (*41*) stated that, while there are strain differences in mammary architecture, no anatomical characteristics have been noted which would indicate that any stock might be susceptible to mammary cancer. Also, the dilute brown mice, used in 2 experiments (*38,40*), have mammary glands of a unique type, "... a type that cannot necessarily be associated with a tendency to have mammary cancer...." (*41*).

In 1946 (*42*), it was reported that, in several inbred strains of mice and their hybrids, no obvious difference could be found in either the amount of lateral budding along the larger ducts or the over-all architecture of the glands, depending upon the presence or absence of MTA in the animal. Also, it was concluded that the development of alveolar hyperplasia was dependent primarily upon the 3 factors associated with the genesis of spontaneous mammary cancer.

To secure additional information about this problem, males of the AZ F_1 generation were studied (*43*), since their female litter mates showed high incidences of mammary cancer when they were observed as either nonbreeders or breeders (*44*). When ovaries were grafted and the agent was injected into 4-week-old castrated AZ F_1 males, a significantly higher incidence of mammary tumors was observed than when the agent was administered to comparable males at 4 months of age. However, animals of the older group did not show a higher incidence than males that were castrated, grafted with ovaries, and injected with MTA when they were 4 months of age. When the average cancer ages were determined from the time following the administration of the agent, they were similar in the 3 groups, regardless of any difference in incidence. It was concluded

that the so-called age factor, or the relative nonsusceptibility of old mice to tumor induction following the introduction of the agent, was not due to the degree of development of the mammary glands in the absence of MTA, at least in these experimental animals.

Various references have been made in this report to the propagation and transmission of the agent by old mice, showing that the activity of the agent usually had not been altered when it was transferred by the experimental animals to their progeny. In other cases, the offspring of cancerous mothers had low incidences of mammary tumors. As cited previously (*34*), the litter in which the mice were born need not influence the genesis of the disease.

Summary

The injection of extracts of transplanted mammary cancer containing the mammary tumor agent (MTA) from either 10^{-1} or 10^{-3} gm. equivalents of tissue produced similar incidences of mammary cancer in young ZBC test animals. A lower incidence resulted when 5×10^{-1} gm. equiv. was administered.

When adult females of the Ax, Zb, and C stocks were injected, the development of mammary cancer could not be correlated with the concentration of the agent in the extract they obtained.

In adult C females, a higher incidence of mammary tumors was observed in those receiving the extract diluted 1,000-fold than in litter mates injected with either 10^{-1} or 5×10^{-1} gm. equiv. of tissue.

Observations on the progeny of injected mothers showed that the development of mammary cancer was influenced by neither the amount of mammary tumor agent administered to the mother nor the litter in which the offspring was born.

The genetic constitution of the progeny born to injected females influenced the incidence and time of development of mammary cancer. In isolated instances, cancerous mothers had offspring with a low incidence of cancer.

Differences in the incidence of mammary cancer in experimental females of various stocks could not be explained by the degree of development of their mammary glands at the time of introduction of MTA.

The injection of extracts containing different amounts of MTA into young C females had little, if any, effect upon the development of mammary cancer in their progeny.

Further studies must be completed in order to understand the relatively greater resistance of older mice to the induction of mammary cancer following the administration of MTA and to ascertain whether it may be correlated with the sensitivity of females of different stocks to male infection with the agent.

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Azaguanine-Deaminase Activity of Several Lymphocytic Leukemias of Mice[1]

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The fundamental biochemical differences between neoplastic cells sensitive to the growth-inhibitory action of chemotherapeutic agents and cells resistant to such action remain largely unknown (*1*). Of great interest, therefore, was the observation of Hirschberg *et al.* (*2*) that several experimental tumors resistant to the action of 8-azaguanine contained an enzyme system capable of deaminating the compound to azaxanthine, a material having no carcinostatic action, while 3 of 4 tumors susceptible to azaguanine were low in this enzyme. It was postulated that the enzymatic control of cellular levels of 8-azaguanine was an important factor in the success or failure of tumor chemotherapy with this drug. Further support for this conclusion was afforded by the finding that a human brain tumor, glioblastoma multiforme, devoid of azaguanine-deaminase activity, was severely damaged by azaguanine in tissue culture. Fetal brain, on the other hand, was less severely affected by azaguanine under the same conditions and showed some enzymatic activity (*3*).

The experimental neoplasms examined by Hirschberg and co-workers, showing high azaguanine-deaminase activity, were innately resistant to azaguanine. Sublines of experimental leukemias in mice have been developed from parent lines sensitive to growth inhibition by azaguanine that are resistant to azaguanine, and, in some instances, dependent on the agent for optimal growth (*4*). Studies on azaguanine-deaminase activity of neoplastic cells were extended in the present report to determine whether the emergence of derived resistance to, or dependence on, azaguanine in experimental leukemias was associated with changes in the azaguanine-deaminase activity of the cells.

Materials and Methods

The neoplasms used were all lymphocytic leukemias, as follows: (*a*) L1210, sensitive to 8-azaguanine; (*b*) L1210/8AG-R, derived from L1210 and resistant to 8-azaguanine (*4*); (*c*) L1210/8AG-D, derived from L1210

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and dependent on azaguanine for optimal growth (*4*); (*d*) L5178;[3] (*e*) L5178/8AG-R, derived from L5178 and resistant to 8-azaguanine; (*f*) L4946, innately resistant to azaguanine.

All of the neoplasms had been transformed to ascitic growths in this laboratory and were used in ascitic form in these experiments. L4946 was carried in (C3Hf × AKR)F_1 mice (*5*); all other neoplasms were carried in (C × DBA)F_1 mice (*5*).

Preliminary determinations to establish satisfactory conditions for the assay of azaguanine-deaminase activity were carried out with livers of normal (C3Hf × AKR)F_1 mice. The conditions finally chosen for tissue preparation were 2-minute homogenization in a pestle homogenizer, using 0.25 *M* sucrose in 0.01 *M* phosphate buffer at *p*H 7.4 as the homogenization medium.

Ten percent homogenates were prepared for liver assays. For determination of enzyme activity in the leukemic cells, ascitic fluids from 2 to 10 mice, killed by cervical dislocation, were pooled and chilled in an ice bath immediately upon withdrawal. The fluids were centrifuged in the cold, the supernatant fluid was discarded, and sucrose medium was added to give approximately a 10 percent suspension of cells. An aliquot of the uniformly suspended cells was added to a Wintrobe tube for determination of the packed-cell volume; the remainder was homogenized for 2 minutes and used for enzyme assays. Similar enzymatic activities were obtained whether homogenization was carried out for 2 or 4 minutes. Microscopic examination of homogenates revealed few unbroken cells. A 2-minute homogenization period was therefore considered adequate.

Enzymatic activity of freshly made tissue preparations was determined by the Warburg technique of Braganca and co-workers (*6*). The main chamber of the Warburg vessels contained 0.5 ml. of 0.15 *M* tris buffer, *p*H 7.4, 1 ml. of azaguanine[4] solution containing 5 mg. per ml. (*2*) (or 1 ml. water in control flasks), tissue homogenate, and sucrose medium to 2.5 ml., when necessary. Unless otherwise indicated, 0.5 ml. of liver homogenates and 1.0 ml. of leukemic-cell homogenates were used for assay. Central wells of the vessels contained 0.2 ml. *N* H_2SO_4 and fluted filter paper. Side arms contained 0.5 ml. of saturated K_2CO_3. Immediately upon addition of tissue preparations to Warburg vessels already containing all other constituents, the vessels were adjusted on manometers and placed in the water bath at 37° C. Stopcocks were closed after 2-minute equilibration, and incubation was allowed to continue for 1 hour, unless otherwise indicated. The alkaline solution was then tipped in from side arms, stopping enzyme activity, and the ammonia released was permitted to diffuse into the central well for an additional 3 hours. At the end of this time, central-well contents were quantitatively withdrawn and diluted to 10 ml. Aliquots were taken for the determination of ammonia by nesslerization (*7*).

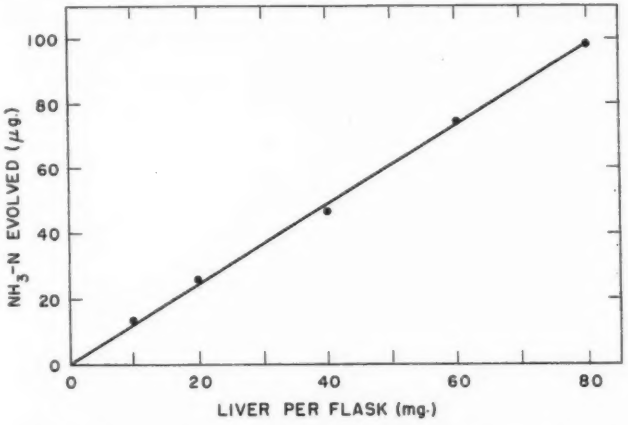
[3] Details regarding this neoplasm, of strain DBA/2 origin, have not been published, but it has been found to be especially sensitive to several purine antagonists, 6-mercaptopurine, 8-azaguanine, and thioguanine. Following inoculation of about 1 million ascitic cells, leukemic deaths occur in DBA/2 or (C × DBA)F_1 mice at 18 days.

[4] Kindly furnished by Lederle Laboratories Division, American Cyanamid Company.

Ammonia produced nonspecifically by homogenates in control flasks was subtracted from ammonia found in azaguanine-containing flasks to give the net ammonia produced through enzymatic activity.[5] Enzyme activity of leukemic cells was calculated as net ammonia nitrogen released per ml. of packed cells per hour.

Results and Discussion

Experiments establishing the validity of the assay procedures employed were carried out with liver homogenates. As shown in text-figure 1, enzymatic activity was proportional to the amount of tissue present over a wide range of concentrations.



TEXT-FIGURE 1.—Azaguanine-deaminase activity of mouse liver as a function of tissue concentration. Incubation was permitted for 1 hour before tipping in K_2CO_3 from side arms.

There was a gradual linear decrease in azaguanine-deaminase activity of mouse liver with time (table 1). Extrapolation of the values shown to zero time gave an azaguanine-deaminase activity of 1.28 mg. NH_3-N per gm. of liver per hour. The average enzymatic activity determined after 1 hour of incubation, therefore, represented about 92 percent of the initial rate of activity.

Recovery of ammonia nitrogen, added to either experimental (with azaguanine) or control (without azaguanine) flasks, was determined following the procedures previously outlined. In 4 determinations, in which the amount of ammonia nitrogen added represented 34 to 68 percent of the total found, recoveries ranged from 96 to 106 percent, with an average recovery of 102 percent.

Azaguanine-deaminase activities of the leukemic cells are presented in table 2. In contrast to the observations made on neoplasms innately

[5] There was no autohydrolysis of the Lederle azaguanine under the conditions employed; correction for a reagent blank was therefore unnecessary.

TABLE 1.—*Azaguanine-deaminase activity of mouse liver as a function of time of incubation*

Time of incubation (*min.*)	Deaminase activity (*mg.NH_3-N/gm.liver/hr.*)
15	1. 26
30	1. 23
60	1. 18
90	1. 12
120	1. 08

resistant to azaguanine (*2*), there was little evidence that leukemic cells showing derived resistance to, or dependence on, azaguanine differed in azaguanine-deaminase activities from the sensitive parent cells. Thus the leukemias of the L1210 lines all showed essentially similar deaminase activities, while L5178/8AG-R cells exhibited only questionably greater activities than the sensitive line.

TABLE 2.—*Azaguanine-deaminase activities of several ascitic lymphocytic leukemias of mice*

Neoplasm	Susceptibility to growth inhibition by azaguanine	Number of experiments*	Azaguanine-deaminase activity (*mg. NH_3-N/ml. cells/hr.*)
L1210	Innate sensitivity	2	0.49±.18†
L1210/8AG-R	Derived resistance	4	0.46±.02
L1210/8AG-D	Derived dependence	6	0.36±.05
L5178	Innate sensitivity	2	0.52±.10
L5178/8AG-R	Derived resistance	2	0.87±.04
L4946	Innate resistance	5	0.49±.08

*Pooled ascitic cells from 2 to 10 mice per experiment.
†Mean ± S.E.

There is indication, moreover, that neoplasms innately resistant to azaguanine need not differ in azaguanine deaminase from susceptible neoplasms. Thus L4946, innately resistant to growth inhibition by azaguanine, exhibited a deaminase activity similar to those of the sensitive lines of L1210 and L5178. It would appear therefore, as pointed out by Hirschberg *et al.* (*2*), that factors other than azaguanine-deaminase activity must be involved in the difference of response of azaguanine-susceptible and azaguanine-resistant neoplasms to the drug.

Summary

Comparisons were made of azaguanine-deaminase activities of several ascitic lymphocytic leukemias of mice. No distinct differences in enzymatic activity were apparent between leukemic lines susceptible to the growth-inhibitory action of azaguanine as compared to sublines of the neoplasms showing either derived resistance to, or dependence on, the agent.

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Distribution of Tumor-Damaging Lignans among Conifers [1]

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It was reported earlier (*1*) that leaves of several species of junipers possess tumor-necrotizing activity for a number of transplantable mouse tumors. In several species, the active material was shown to be podophyllotoxin (*2*) and in one species, a previously undescribed but related lignan, silicicolin (*3*), now called deoxypodophyllotoxin (*4*).

Further search for tumor-necrotizing substances in the leaves of conifers has revealed the presence of species in which active materials are present in 4 additional genera, *Libocedrus*, *Podocarpus*, *Chamaecyparis*, and *Callitris*.

This paper reports the isolation of podophyllotoxin and deoxypodophyllotoxin from additional species of *Juniperus*, the distribution of tumor-damaging activity among the families Ginkgoaceae, Taxaceae, Pinaceae, and Casuarinaceae, and the isolation and identification of active lignans from one species each of *Libocedrus* and *Chamaecyparis*.

Methods

The testing of the conifers was carried out as described previously(*1*). An aqueous suspension of dried, powdered leaves was injected in a dose of 1 mg. per gm. subcutaneously into CA F_1 mice bearing sarcoma 37. The effect on the tumor was evaluated as before. (*See* footnote to table 1.)

For the fractionation of the leaves of the conifers (and, in one case, the berries) the dried material was finely ground before extraction. It was found convenient to use a commercial-model coffee mill set for the finest grind. The procedure employed for the juniper leaves, involving solvent partition and chromatography on activated alumina was essentially that previously described (*2*). With some modification noted in the text,

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this procedure was also followed for the fractionation of juniper berries. Leaves of the *Chamaecyparis* species and the *Libocedrus* species required variation of the procedure with additional chromatography and solvent partition. Examples of these are given in the text.

In a number of instances, where the aqueous suspension of ground leaves showed questionable activity, an attempt was made to concentrate the tumor-damaging substances by acetone extraction. The active material, if present, appeared in the acetone-soluble fraction. By this procedure it was possible to concentrate the activity approximately fivefold and thus verify the presence or absence of activity in questionable cases.

Experimental Results

The distribution of tumor-necrotizing activity in the leaves of conifers is shown in table 1. The taxonomic classification followed is that of Dallimore and Jackson (*5*) for the 3 families of conifers Ginkgoaceae, Taxaceae, and Pinaceae. A fourth family, Casuarinaceae, commonly known as Australian pine, was included because of a superficial resemblance of the members of this family to pines. Although the trees bear cones, they are not gymnosperms. In this family, which is represented by a single genus, *Casuarina*, the trees are leafless but the green branchlets resemble pine needles.

Since many of the species of conifers are dioecious, both male and female plants were tested whenever possible. This is noted in table 1.

Geographic location is included because of an indication noted in previous work (*1*) that this plays a part in the degree of activity of the leaves.

Relationship of Tumor-Damaging Activity and Botanical Classification

Ginkgoaceae.—In the monotypic family Ginkgoaceae leaves of the sole species, *Ginkgo biloba*, were inactive.

Taxaceae.—Thirty-two species, representing 8 genera of the family Taxaceae, were tested. Male and female plants of a single species, *Podocarpus totara*, alone showed activity. However, another sample from the same source in New Zealand was inactive. A sample from Australia was also inactive.

Pinaceae.—Members of 26 genera, including over 100 species of the family Pinaceae were tested. Tumor-necrotizing activity was found to be confined to 4 genera, *Juniperus*, *Libocedrus*, *Chamaecyparis*, and *Callitris*, all in the cypress tribe (Cupressineae).

In Bailey's classification of the genera of conifers (*6*) emphasis is placed on leaf structure as a major taxonomic criterion. When the species of junipers tested are listed (table 2) according to the taxonomic sequence of Bailey, all of the active species fall into a single subgroup, D.D.

The classification of the genus *Libocedrus* according to Bailey does not include all the species tested in the present work, but it is apparent in table 3 that the active species are closely related taxonomically. This also holds true if the plants are grouped according to the proposed reclassi-

TABLE 1.—*Distribution of tumor-damaging activity among species of conifers.* [*Approximately 40 species of conifers reported previously are not included in this list.* See *ref.* (1).]

The footnotes to this table appear at the end of the table on p. 91.

Botanical and common names	Source	Location and sex	Activity †
Family: GINKGOACEAE			
Ginkgo biloba L., Ginkgo or maidenhair tree	5	Mass. ♂	—
Family: TAXACEAE			
I. The yew tribe: Taxeae			
Cephalotaxus drupacea S. & Z.	5	Md. (berries —)	—
Dacrydium bidwillii Hook. f., Mountain pine	7	N. Z. ♂ & ♀	—
" *biforme* Pilger, Manoao	7	N. Z. ♂ & ♀	—
" *colensoi* Hook., Westland pine	7	N. Z. ♂ & ♀	—
" *cupressinum* Soland., Rimu	7	N. Z. ♂ & ♀	—
" *franklinii* Hook. f., Huon pine	1a	Tasmania	—
" *intermedium* T. Kirk, Yellow silver pine	7	N. Z. ♂ & ♀	—
" *kirkii* F. v. Muell., Manoao	7	N. Z. ♂ & ♀	—
Pherosphaera hookeriana Archer	1a	Tasmania	—
Phyllocladus alpinus Hook. f., Alpine celery-topped pine	7	N. Z. ♂ & ♀	—
" *glaucus* Carr., Toatoa	7	N. Z. ♀	—
" *rhomboidalis* Rich., Celery-topped pine	1a	Tasmania	—
" *trichomanoides* D. Don, Tanekaha	7	N. Z.	—
Taxus baccata var. *repandens* Hort., Spreading yew	14	Wash., D. C.	—
" *brevifolia* Nutt., Pacific yew	3	Wash. State	—
" *cuspidata* S. & Z., Erect yew	14	Wash., D. C.	—
Torreya californica Torrey, California nutmeg	4	Calif. ♂	—
" *californica* " " "	1b	Australia	—
" *nucifera* (L.) S. & Z., Kaya	1b	Australia	—
II. The podocarpus tribe: Podocarpeae			
Microcachrys tetragona Hook. f.	1a	Tasmania	—
Podocarpus acutifolius T. Kirk	7	N. Z.	—
" *alpinus* R. Br., Mountain plume pine	1c	Australia	—
" *alpinus* " " " " "	1a	Tasmania	—
" *amarus* Blume, Black pine	2a	Australia (2 samples)	—

TABLE 1.—*Distribution of tumor-damaging activity among species of conifers.* [*Approximately 40 species of conifers reported previously are not included in this list.* See *ref.* (1).]—Continued

The footnotes to this table appear at the end of the table on p. 91.

Botanical and common names	Source	Location and sex	Activity †
Family: TAXACEAE—Continued			
Podocarpus andinus Poepp., Plum-fruited yew	1b	Australia	−
" *andinus* " " " "	2b	"	−
" *dacrydioides* A. Rich, Kahikatea	7	N. Z. ♂ & ♀	−
" *elatus* R. Br., She-pine	1b	Australia (3 samples)	−
" *elatus* " " " "	2a		
" *elatus* " " " "	2b		
" *falcatus* (Thunb.) R. Br., Oteniqua yellow wood	1d	Australia	−
" *ferrugineus* D. Don, Miro	7	N. Z. ♀	−
" *macrophyllus* D. Don, Kusamaki	5	Mass.	−
" *nivalis* Hook. f., Alpine totara	1d	Australia	−
" *sellowii* Klotzsch, Pinheira brava	13	Brazil	−
" *spicatus* R. Br., Matai	7	N. Z. ♂ & ♀	−
" *totara* D. Don, Totara	7	N. Z. ♂ & ♀	+
" *totara* " "	7	N. Z.	−
" *totara* " "	1b	Australia	−
Family: PINACEAE			
I. The cypress tribe: Cupressineae			
Actinostrobus pyramidalis Miq	1f	Australia	−
Callitris arenosa A. Cunningham, Cypress pine	1d	Australia (3 samples)	−
(syn. *C. columellaris*)	2a		
" *calcarata* R. Br., Black cypress pine	1c	Australia	−
	2a		
" *drummondii* (Parl.) F. v. Muell	1f	Australia (2 samples)	++
" *glauca* R. Br., Murray River pine	1c	Australia (4 samples)	−
	1f		
	2a		

" *intratropica* Benth. & Hook. f.	1e	Australia	+
" " " "	1e	Australia	−
" *macleayana* Benth. & Hook. f.	2c	Australia	−
" *morrisonii* R. T. Baker, Morrison's cypress pine	1f	Australia	−
" *muelleri* Benth. ex F. v. Muell., Illawarra pine	2c	Australia	−
" *oblonga* Rich., Tasmanian cypress pine	1a	Tasmania	−
" *propinqua* R. Br.	2d	Australia	−
" *rhomboidea* R. Br., Illawarra mountain pine	2a	Australia (3 samples)	−
	2c		
" *robusta* R. Br., Common cypress pine	1f	Australia	−
" *tasmanica* Baker & Sm., Oyster Bay pine	1a	Tasmania	−
	1c		
" *verrucosa* R. Br., Turpentine pine	1c	Australia (4 samples)	−
	2c		
	1f		
Chamaecyparis lawsoniana (Murr.) Parl., Lawson cypress	5	Mass. (cones ±) ‡	±
" *obtusa* (S. & Z.) Endl., Hinoki cypress	15	Wash., D. C	±
" *pisifera* var. *squarrosa* Beissn. & Hochst., Moss cypress	14	Wash., D. C	−
Cupressus benthamii Endl., Bentham cypress	5	Cuba	−
" *forbesii* Jepson	4	Calif	−
" *glabra* Sudworth, Smooth cypress	4	Calif	−
" *lusitanica* Mill., Mexican cypress	5	Cuba	−
" *macnabiana* Murr., MacNab's cypress	4	Calif	−
" " (syn. *C. nevadensis* Abrams)	5	Cuba	−
" *macrocarpa* Hartweg, Monterey cypress	15	Calif	−
" *macrocarpa* Hartweg	5	Cuba	−
" *sempervirens* var. *stricta* Ait., Columnar Italian cypress	5	Cuba	−
Diselma archeri Hook. f.	1a	Tasmania	−
Juniperus barbadensis L., Barbados juniper	5	Florida ♂ & ♀ ‡	−
" *cedrus* var. *maderensis*, Canary Islands juniper	4	Madeira	−
" *horizontalis* Moench, Creeping juniper	6	Wis. ♀ (berries +)	++
" *japonica* Carr., Japanese juniper	4	Calif	−
" *lucayana* Britt., West Indies cedar	15	Florida	++
" *lucayana* " " " "	5	Cuba ♀	−
" *monosperma* Sarg., Cherrystone juniper	10	Nevada ♀	−
" *monosperma* " " "	5	Cuba	−
" *procera* Hochst., African juniper	4	Africa	−
" *squamata* var. *meyeri* Rehd., Meyer juniper	14	Wash., D. C	−
" *utahensis* (Engelm.) Lemmon, Utah juniper	8	Calif. ♀	−

TABLE 1.—*Distribution of tumor-damaging activity among species of conifers.* [*Approximately 40 species of conifers reported previously are not included in this list.* See *ref.* (1).]—Continued

The footnotes to this table appear at the end of the table on p. 91.

Botanical and common names	Source	Location and sex	Activity †
Family: PINACEAE—Continued			
Juniperus virginiana var. *burkii* Hort	9	Mass. ♀	−
" " var. *canaertii* Sénéclauze	9	Mass. ♀	±
" " var. *cinerascens*	9	Mass. ♀	++
" " var. *crebra* Fern. & Grisc	9	Mass. ♀	++
" " var. *elegantissima* Hochst	9	Mass	+
" " var. *fastigata*	9	Mass. ♀	++
" " var. *glauca* Carr	9	Mass. ♀	+
" " var. *globosa* Beissn	9	Mass. ♂ & ♀	+
" " var. *kosteri* Beissn	9	Mass. ♂ & ♀	++
" " var. *pendula* Beissn	9	Mass. ♂	++
" " var. *pendula* "	9	Mass	−
" " var. *plumosa* Rehd	9	Mass. ♀	+
" " var. *pyramidalis* Carr	9	Mass. ♂	+
" " var. *pyramidiformis hilli* D. Hill, Dundee juniper	9	Mass. ♂	+
" " var. *reptans* Beissn	9	Mass. ♀	+
" " var. *schotti* Gord	9	Mass. ♀	±
Libocedrus arfakensis Gibbs	9	Dutch New Guinea, § ‖	−
" *bidwillii* Hook. f., Pahautea	7	N. Z.	++
" *chilensis* (D. Don) Endl., Chilean incense cedar	4	Calif	++
" *chilensis* " " " "	11	Oregon	++
" *chilensis* " " " "	12	Chile	++
" *decurrens* Torr., Incense cedar	4	Calif. ‖	−
" *formosana* Florin, Formosan incense cedar	4	Calif. ‖	−
" *macrolepis* Benth. & Hook	9	Formosa § ‖	−
" *papuana* F. v. Muell	9	New Guinea § ‖	−
" *plumosa* (D. Don) Sarg., Kawaka	7	N. Z.	++
" *torricellensis* Schlect	9	New Guinea § ‖	−
" *uvifera* (D. Don) Pilger (*Pilgerodendron uviferum*)	9	Ireland § ‖ (at 2 mg./gm.++)	±
Tetraclinis articulata (Vahl) Mast., Arar tree	5	Florida	−
" *articulata* " " " "	5	Cuba	−
" *articulata* " " " "	1d	Australia	−

Thuja plicata D. Don, Giant arbor-vitae	4	Calif.	–
" *standishii* (Gord.) Carr., Japanese or Standish arbor-vitae	5	Mass.¶	–
Widdringtonia sp. (juvenile foliage)	5	Calif.	–
" " " "	5	Calif.	–
II. The taxodium tribe: Taxodineae			
Athrotaxis cupressoides D. Don, Tasmanian cedar	1a	Tasmania	–
" *selaginoides* D. Don, King William pine	1a	Tasmania	–
Cunninghamia lanceolata (Lamb.) Hook. f., China fir	4	Calif.	–
Metasequoia glyptostroboides Hu & Cheng	5	Wash., D. C.	–
Sciadopitys verticillata S. & Z., Japanese umbrella pine	1d	Australia	–
Sequoia sempervirens Endl., Redwood	4	Calif.	–
Taiwania cryptomerioides Hayata	1d	Australia	–
" *flousiana* Gaussen (juvenile foliage)	4	Calif.	–
Taxodium distichum (L.) Rich., Common bald cypress	5	N. C.	–
" *mucronatum* Ten., Mexican cypress	1b	Australia	–
III. The fir tribe: Abietineae			
Abies cephalonica Loud., Grecian fir	1d	Australia	–
" *grandis* Lindl., Giant fir	3	Wash. State	–
" *nordmanniana* Spach, Nordmann fir	15	Maryland	–
Keteleeria fortunei (A. Murr.) Carr.	1b	Australia	–
Larix laricina (Du Roi) K. Koch, American larch	14	Wash., D. C	–
Picea abies (L.) Karst, Common spruce	1d	Australia	–
" *albertiana* S. Brown, Alberta spruce	14	Wash., D. C	–
" *glauca* (Moench) Voss, White spruce	1d	Australia	–
" *pungens* Engelm., Blue spruce	1d	Australia	–
" *sitchensis* (Bong.) Carr., Sitka spruce	1d	Australia	–
Pinus canariensis C. Smith, Canary Islands pine	1d	Australia	–
" *montezumae* Lambert, Rough-branched Mexican pine	1d	Australia	–
" *occidentalis* Swartz	5	Cuba	–
" *patula* Schlecht. & Cham., Spreading-leaved pine	1b	Australia	–
" *roxburghii* Sarg., Long-leaved Indian pine	1b	Australia	–
" *sabiniana* Douglas, Digger pine	5	Calif.	–
" *tenuifolia* Benth.	1b	Australia	–
" *wallichiana* A. B. Jacks., Bhutan pine	1b	Australia	–
Pseudotsuga macrocarpa Mayr. Bigcone spruce	4	Calif. ¶	–
" *taxifolia* (Poir.) Britt., Douglas fir	3	Wash. State	–
Tsuga heterophylla (Raf.) Sarg., Western hemlock	3	Wash. State	–

TABLE 1.—*Distribution of tumor-damaging activity among species of conifers.* [*Approximately 40 species of conifers reported previously are not included in this list.* See *ref.* (1).]—Continued

Botanical and common names	Source	Location and sex	Activity †
Family: PINACEAE—Continued			
IV. The araucaria tribe: Araucarineae			
Agathis australis Salisb., Kauri pine	7	N. Z.	—
" *loranthifolia* Salisb. Amboyna pitch tree	1b	Australia	—
" *moorei* (Lindl.) Mast.	1b	Australia	—
" *obtusa* (Lindl.) Mast.	1b	Australia	—
" *ovata* (C. Moore) Warburg	1b	Australia	—
" *palmerstonei* F. v. Muell., North Queensland Kauri pine	2a	Australia (2 samples)	—
" *robusta* (C. Moore) F. M. Bail., South Queensland Kauri pine	2a	Australia	—
" *spinulosa* Warburg	1b	Australia	—
" *vitiensis* (Seem.) B. & H., Fijian Kauri pine	1b	Australia	—
Araucaria angustifolia O. Kuntze, Brazilian candelabra tree	13	Brazil	—
" *araucana* (Molina) K. Koch., Monkey puzzle	1d	Australia	—
" *bidwillii* Hook., Bunya pine	2a 2b	Australia	—
" *columnaris* Hook., Cook's pine	1b 2b	Australia	—
" " (syn. *A. cookii* R. Br.)	1d	Australia	—
" *cunninghamii* Sweet, Hoop pine	2a 2b	Australia	—
" *heterophylla* (Salisb.) Franes.	1b	Australia	—
Family: CASUARINACEAE (Australian pines, Beefwood)			
Casuarina angulata Poiss.	5	Cuba	—
" *distyla* Vent., Shrubby sheoke	1c	Australia	—
" *equisetifolia* L.	5	Florida	—
" *fraseriana* Miq.	5	Cuba	—
" *lepidophloia* F. v. Muell.	5	Cuba (2 samples)	—
" " F. v. Muell., Belar	1c	Australia	—
" *luehmanii* R. T. Baker, Buloke	1c	Australia	—

"	*muelleriana* Miq., Slaty sheoke	1c	Australia (2 samples)	+
"	*pusilla* Macklin, Dwarf sheoke	1c	Australia	−
"	*stricta* Ait., Drooping sheoke	1c	Australia	−
"	*suberosa* Ott. & Dietr., Black sheoke	1c	Australia	−

*Sources:

1. Dr. J. R. Price, C. S. I. R. O., Melbourne, Australia, with the cooperation of:
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15. Collected by one of the authors (D. B. F.).

†++Marked tumor damage in 80–100 percent of animals from a single dose of 1 mg./gm.
+Appreciable tumor damage in at least 50 percent of the animals.
±Questionable effect.
−No appreciable tumor damage.
‡Concentration with acetone gives positive results.
§Dried herbarium specimen.
‖See table 4 for botanical name in recent reclassification by Li.
¶Concentration with acetone gives negative results.

TABLE 2.—Juniperus [*according to Bailey* (6)]

A. True junipers	
(Leaves needlelike and in 3's, jointed or definitely articulated to the twig.)	
cedrus	neg.
communis	neg.
oxycedrus	neg.
rigida	neg.
A.A. Savin junipers	
(Leaves scalelike and opposite or needlelike and in 3's, not jointed to the twig but decurrent on it.)	
B. Leaves needlelike	
procumbens	neg.
recurva	neg.
squamata	neg.
B.B. Leaves scalelike and opposite or needlelike on certain growths.	
C. Margin of leaves denticulate or fringed	
californica	neg.
monosperma	neg.
occidentalis	neg.
utahensis	neg.
C.C. Margin of leaves entire	
D. Color of fruit brown or purplish-brown	
chinensis	neg.
excelsa	neg.
japonica	neg.
procera	neg.
D.D. Color of fruit blue	
barbadensis	pos.
lucayana	pos.
silicicola	pos.
virginiana	pos.
scopulorum	pos.
horizontalis	pos.
sabina	pos.

fication of the genus by Li (7). In table 4 we see that the 3 species retained in the genus *Libocedrus* are active, while those in the proposed new genera *Heyderia* and *Papuacedrus* are inactive. The single species of the new genus *Pilgerodendron* was moderately active.

Aqueous suspensions of ground needles of *Chamaecyparis lawsoniana* and *C. obtusa* showed questionable activity at a dose of 1 mg. per gm. Concentration of the material from *C. lawsoniana* by acetone extraction yielded a fraction showing high tumor-damaging activity at a dose of 1 mg. per gm. Our sample of *C. obtusa* was insufficient for further testing by extraction.

C. thyoides and *C. nootkatensis*, which had no activity by the usual test, were also checked by extraction with acetone. In these cases no activity

TABLE 3.—Libocedrus [*according to Bailey* (6)]

A. Facial or median leaves equaling or perhaps slightly exceeding the lateral leaves, all dark green.	
decurrens	neg.
formosana	neg.
macrolepis	neg.
A.A. Facial leaves much smaller and usually shorter than lateral ones, the latter with white lines underneath.	
bidwillii	pos.
chilensis	pos.
plumosa	pos.

TABLE 4.—Libocedrus [*according to Li* (7)]

Genus *Heyderia*	
1. *Heyderia decurrens* (Torr.) K. Kock (*Libocedrus decurrens* Torr.).	neg.
2. *Heyderia formosana* (Florin) Li (*Libocedrus formosana* Florin).	neg.
3. *Heyderia macrolepis* (Kurz) Li (*Libocedrus macrolepis* Benth. & Hook.).	neg.
Genus *Pilgerodendron*	
1. *Pilgerodenron uviferum* (D. Don) Florin (*Libocedrus uvifera* Pilger).	pos.
Genus *Libocedrus*	
1. *Libocedrus plumosa* (D. Don) Sarg	pos.
2. *Libocedrus bidwillii* Hook, f	pos.
3. *Libocedrus chilensis* (D. Don) Endl	pos.
Genus *Papuacedrus*	
1. *Papuacedrus papuana* (F. v. Muell.) Li (*Libocedrus papuana* F. v. Muell.).	neg.
2. *Papuacedrus torricellinsis* (Schlect.) Li (*Libocedrus torricellinsis* Schlect.).	neg.
3. *Papuacedrus arfakensis* (Gibbs) Li (*Libocedrus arfakensis* Gibbs).	neg.

was found in the acetone-soluble fraction. These results are shown in table 5, in which the species are listed in the sequence of Bailey's classification. In this genus also the active members appear to be closely related species.

TABLE 5.—Chamaecyparis [*according to Bailey* (6)]

A. Leaves on the sides and on the faces of the branchlets of practically equal size.	
thyoides	neg. (acetone-sol. neg.)
pisifera	neg.
nootkatensis	neg. (acetone-sol. neg.)
A.A. Leaves on the 2 sides of the twig distinctly larger than those on the faces, the body of the leaf closely appressed.	
obtusa	questionable activity
lawsoniana	questionable activity (acetone-sol. pos.)

It should be noted that, since it was not possible to check all "negative" plants by the acetone-extraction procedure, some plants may have contained active material, but in too small amount to be detected by the original screening method.

Nine species of the single genus *Casuarina* in the family Casuarinaceae (Australian pine), were tested. The needles of only 1 species, *C. muelleriana*, showed slight activity at a dose of 1 mg. per gm.

Fractionation Methods

To supplement our knowledge of the distribution and identity of tumor-damaging lignans in junipers, leaves from male and female trees of various species were fractionated separately. In one case, *Juniperus sabina* var. *tamariscifolia*, the active lignan was isolated from the berries.

The usual procedure for the leaves of junipers, previously described (*2*), was modified for the fractionation of the berries. Successive extraction with acetone, ligroin, and absolute ethanol, followed by chromatography on activated alumina with absolute ethanol, yielded an active crystalline

compound, identified as deoxypodophyllotoxin by melting point (166.4–167.1° C. corr.), mixed melting point (167.2–168.1° C. corr.), and infrared absorption spectrum. Further treatment of the mother liquor with ethyl acetate, additional chromatography, elution with ethyl acetate, and crystallization from ethanol increased the yield of deoxypodophyllotoxin to 0.3 percent. This accounted for 75 percent of the activity, estimated by bioassay of the original ground berries. A previous fractionation of leaves from the female plant of this species yielded a crystalline fraction thought at first (*2*) to be a new substance, but later, on recrystallization, was found to consist at least in part of deoxypodophyllotoxin.

Table 6 presents the species of plants fractionated, the sex of the plant when known, and the identity and yield of the lignans isolated from these plants.

Fractionation of the leaves of *Libocedrus chilensis* was carried out in the following manner. Acetone extraction followed by ligroin extraction of the acetone-soluble portion yielded a ligroin-insoluble portion containing essentially all of the activity. Chromatography on activated alumina of an absolute-ethanol solution of this fraction yielded a very small cut of extremely active material. A second cut, obtained by elution with 70 percent alcohol, was relatively inactive. The active fraction could not be partitioned satisfactorily by the solvents employed for the isolation of the active substance from junipers. Accordingly, the following modification was employed:

Benzene extraction gave 75 percent of benzene-soluble substance, containing the greater part of the activity. Chromatography of a benzene solution of this fraction on activated alumina yielded the following cuts: 1) Benzene elution; the first cut was terminated at the end of the yellow

TABLE 6

Substance	Lignan isolated	Yield (*percent*)
Juniperus lucayana, leaves	podophyllotoxin	0. 10
J. scopulorum, leaves	podophyllotoxin	0. 17
J. scopulorum, leaves from ♂ tree	podophyllotoxin	0. 14
J. silicicola, leaves	deoxypodophyllotoxin	0. 11
J. silicicola, leaves from ♂ tree	podophyllotoxin	0. 04
J. sabina (dried savin tops)	podophyllotoxin	0. 20
	savinin	0. 12
J. sabina var. *tamariscifolia*		
leaves from ♂ plant	podophyllotoxin	0. 14
leaves from ♀ plant	deoxypodophyllotoxin	0. 10
berries from same ♀ plant	deoxypodophyllotoxin	0. 30
wood from same ♀ plant (inactive)	none	
J. virginiana, leaves from ♂ tree	podophyllotoxin	0. 05
	picropodophyllin	trace
J. virginiana, leaves from ♀ tree	podophyllotoxin	0. 10
berries from same ♀ tree (inactive)	none	
Libocedrus chilensis, leaves (monoecious)	deoxypodophyllotoxin	0. 03
Chamaecyparis lawsoniana, leaves (monoecious).	deoxypodophyllotoxin	0. 15

band on the column. A yellow, waxy pigment was obtained, soluble in chloroform and ether, which faded on standing, possibly a carotenoid; it had no effect on sarcoma 37 at 64 γ per gm.

2) Further elution with benzene until the eluate was practically colorless and gave little residue on drying constituted the second cut. This cut was highly active.

3) A third cut, obtained by elution with 5 percent absolute ethanol : 95 percent benzene, yielded an active fraction that accounted for 99 percent of the original sample.

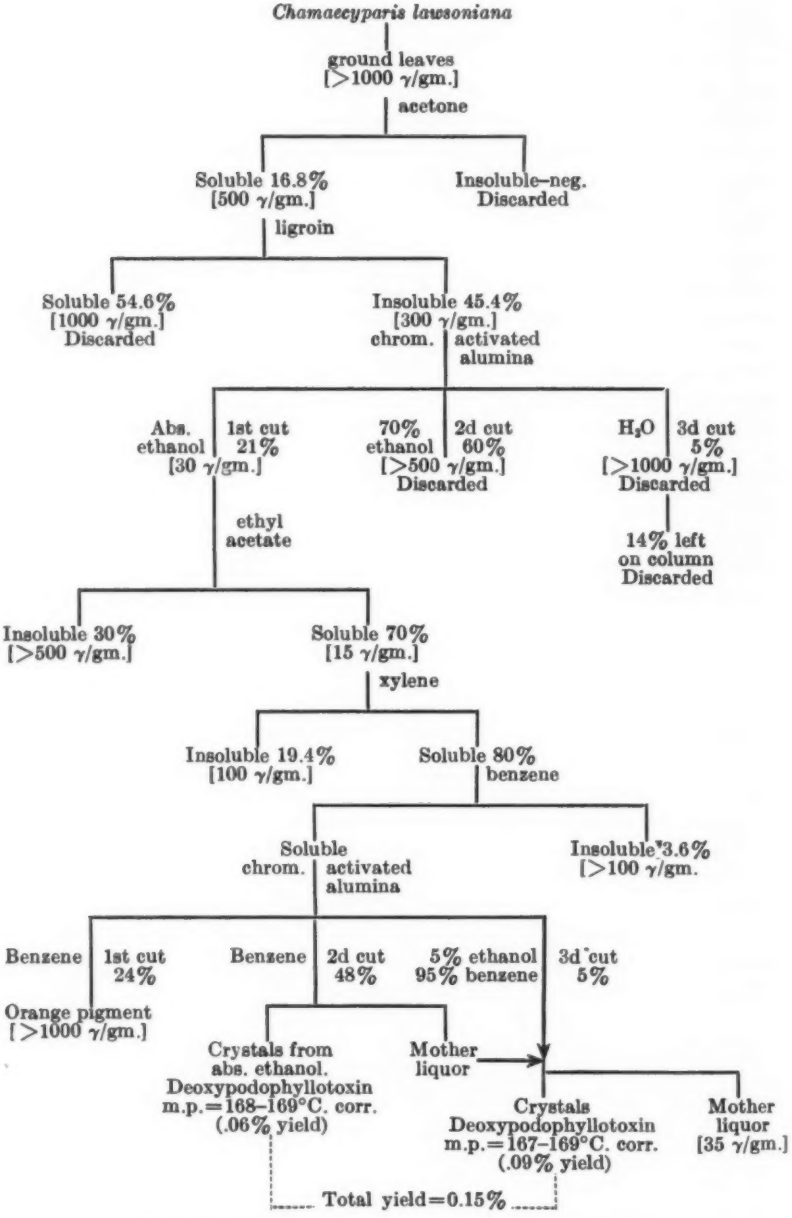
The second cut, when taken up in absolute ethanol, yielded colorless, inactive crystals (m.p. 74 to 76° C.) This compound was not a lignan according to infrared spectra and therefore was not identified further.[4] A second crop of colorless crystals, rhombic prisms, was purified by recrystallization from methanol (m.p. 165–167° C.) and identified as deoxypodophyllotoxin. A third crop yielded colorless, low-melting inactive crystals, non-lignan in structure.

The third cut was treated in the same manner and yielded an additional crop of deoxypodophyllotoxin.

Chamaecyparis lawsoniana leaves were fractionated by a modification of the procedure used for the junipers. Due to the low content of active material, it was necessary to rechromatograph on activated alumina, eluting with benzene, in order to concentrate the material after the extraction with xylene. The first cut, representing 24 percent of the chromatographed sample, was bright orange in color and showed no tumor-damaging activity at 1 mg. per gm. Unlike the yellow pigments found in *Libocedrus* leaves, this material did not lose color on standing. No attempt was made to purify or identify this inactive compound. A second benzene cut, representing 48 percent of the sample, was taken up in absolute ethanol and deposited fine, colorless crystals. Purification and identification of the crystals by melting point (168–169° C. corr.), mixed melting point (168.2–168.8° C. corr.), and infrared absorption spectrum showed the compound to be deoxypodophyllotoxin. The mother liquor was combined with the small third cut and taken up in absolute ethanol, yielding additional crystals of deoxypodophyllotoxin and bringing the yield to 0.15 percent. This constitutes 100 percent recovery based on bioassay of the acetone-soluble fraction. The original ground needles before extraction, when tested at the highest dose of 1 mg. per gm., had given questionable results.

Text-figure 1 outlines the fractionation procedure employed for *Chamaecyparis lawsoniana*. The bioassay value (minimal effective dose) is given in brackets. The apparent increase in potency with increasing purification of the active material from *C. lawsoniana* might be attributed to variation between successive bioassays. However, it seems more probable that this effect is due to the removal of extraneous substances, especially the waxy materials, which may retard the absorption of the active substance sufficiently to give a delayed effect on the tumors.

[4] Probably an "etholide" [*see* ref. (8), footnote 7].



TEXT-FIGURE 1.—Fractionation procedure employed for *Chamaecyparis lawsoniana*.

Discussion

Various lignans have been isolated from the heartwood of conifers. This literature has been summarized by Erdtman (*8*) in his review of the subject (1952). Aside from studies on their volatile oil content, the leaves have received less attention. Prior to the earlier reports from this laboratory (*2,3*) there seems to have been no recognition of the presence of lignans in the leaves of the conifers.

In the present work, the list of conifers which contain lignans in their leaves has been extended. Since we have been interested primarily in the detection of tumor-damaging materials, we have followed the fractionation of the lignans by a bioassay for tumor-damaging activity. This has necessarily resulted in the loss of most inactive lignans, possibly widespread in the leaves.

Thus far, the active lignans have proved to be either podophyllotoxin or deoxypodophyllotoxin. During the isolation of podophyllotoxin from the leaves of *Juniperus sabina* (*2*), a second lignan, inactive against tumors, was found. This lignan was named savinin. Recently, Masumura and Okumura reported (*9*) the isolation of the same compound, which they named hibalactone, from the young leaves of "nikohiba" (a garden variety of *Chamaecyparis pisifera*) and of "chyabohiba" (a garden variety of *C. obtusa*), while Kariyone and Isoi (*10*) found it in the leaves of 2 varieties of *C. obtusa*, Hinoki cypress (*C. obtusa* Endl.) and "chabo-hiba" (*C. obtusa* S. & Z. var. *breviramea* Mast.).

Kariyone and Isoi (*10*) isolated savinin from the ligroin-soluble fraction of *C. obtusa* and did not investigate the ligroin-insoluble fraction; while we found that the ligroin-soluble fractions of the conifers were essentially inactive and that the tumor-damaging lignans, podophyllotoxin and deoxypodophyllotoxin, were to be found in the ligroin-insoluble fractions. Thus, one would expect Kariyone and Isoi to overlook any of the tumor-damaging lignans mentioned, while we would miss any savinin. Since it was noted in the present study that our sample of *C. obtusa*, insufficient in quantity for further study, showed slight tumor-damaging activity, it is not improbable that this plant also contains podophyllotoxin or deoxypodophyllotoxin. Moreover, *C. lawsoniana*, a closely related species, was definitely shown to contain deoxypodophyllotoxin.

In the course of the isolation of podophyllotoxin from the leaves of the male plant of *Juniperus virginiana*, a trace of the inactive lignan picropodophyllin was found in the final mother liquor. Picropodophyllin was first isolated and identified by Podwyssotzki (*11*) from *Podophyllum peltatum*. From later work on the isolation of lignans from this same plant it is evident that the podophyllotoxin, present in large amounts, had been converted to picropodophyllin by the alkaline treatment during the extraction process. A recent review of the chemistry of podophyllin by Hartwell (*12*) discusses this subject more thoroughly. It is possible that the trace of picropodophyllin found in *J. virginiana* may also be an artifact.

In both of the monoecious plants tested, *Libocedrus chilensis* and

Chamaecyparis lawsoniana, the leaves contained deoxypodophyllotoxin. Leaves of a male plant of *Juniperus sabina* var. *tamariscifolia* contained podophyllotoxin, while the leaves of the female plant contained deoxypodophyllotoxin. The berries from this same female plant contained deoxypodophyllotoxin, whereas the wood gave a negative test for active lignans. Leaves from both the male and female plants of *J. virginiana* contained podophyllotoxin, whereas the berries from the same female plant were inactive.

Deoxypodophyllotoxin was isolated from *J. silicicola* of unknown sex. A known male plant yielded podophyllotoxin.

Only 1 plant of the family Taxaceae, *Podocarpus totara*, showed activity. Extraction procedures used for the isolation of the active species of Pinaceae were not applicable in this case. Although the active tumor-damaging substance has not yet been isolated, it may prove to be a lignan. In favor of this possibility is the report of the presence of 2 lignans, matairesinol and conidendrin, in the heartwood of *Podocarpus spicatus* (*13*,*14*).

Matairesinol and conidendrin are inactive against sarcoma 37 (*15*). No attempt has been made to determine whether these inactive lignans are present in the leaves of conifers.

On the other hand, the fact that the tumor-damaging activity of *P. totara* is not concentrated by acetone, unlike the case with other active leaves wherein the activity is concentrated, suggests the possibility of a different chemical structure for the active substance. This possibility is being investigated.

Casuarina muelleriana (Fam. Casuarinaceae), an evergreen tree resembling the conifers, was found to be the only species of 9 of this genus tested whose leaves showed tumor-damaging activity. Extraction procedures used for the conifers were not satisfactory in this case. It is planned to study this case further.

Summary

The leaves of 153 species and varieties of conifers grouped into 36 genera have been studied for the presence of lignans damaging to sarcoma 37 in mice. Of these, 5 genera are newly reported to show tumor-damaging activity; these are *Libocedrus* (Pinaceae), *Chamaecyparis* (Pinaceae), *Callitris* (Pinaceae), *Podocarpus* (Taxaceae), and *Casuarina* (Casuarinaceae). In addition, several new species of the previously reported *Juniperus* (Pinaceae) were found to possess tumor-damaging activity.

Isolation and identification of tumor-damaging lignans from the active species that were fractionated yielded podophyllotoxin and deoxypodophyllotoxin. *Callitris* and *Podocarpus* were not studied in this way.

In the dioecious genus *Juniperus*, leaves of known male plants yielded podophyllotoxin, while leaves of known female plants gave either podophyllotoxin or deoxypodophyllotoxin, depending on species.

In 1 species of *Juniperus*, leaves of the male plant yielded podophyllotoxin, while leaves of the female plant yielded deoxypodophyllotoxin.

The berries from the same female plant were active and gave deoxypodophyllotoxin, while the wood was inactive.

Leaves of 2 monoecious species of *Libocedrus* and *Chamaecyparis* yielded deoxypodophyllotoxin only.

Species possessing tumor-damaging activity were found to be closely grouped according to botanical classification.

In certain species, marked differences noted in the degree of tumor-damaging activity were believed due possibly to differences in geographic location.

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Carcinogenicity of Some Ingested Acetylated Mono- and Diaminobiphenyl Compounds in the Rat [1,2]

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The role in chemical carcinogenesis of the methylene bridge at the 9-position of *N*-2-fluorenylacetamide has been studied by investigators in at least 2 laboratories (*1–5*). Miller *et al.* (*3*) concluded that strong carcinogenic activity for the rat liver is dependent on the presence of a fluorene or modified fluorene nucleus with an intact methylene bridge. The biphenyl nucleus (lacking the methylene bridge at the 9-position), however, appeared to them to be an equally active structural unit for mammary carcinogenesis, while the structural requirements for induction of tumors of the acoustic duct and the epithelium of the small bowel were considered to be intermediate between those of the liver and mammary gland.

Walpole *et al.* (*6*) report that 3,2′-dimethylaminobiphenyl administered subcutaneously in arachis oil in comparatively low doses induces a high incidence of intestinal tumors in male Wistar rats. They (*7*) reported no tumors of the liver by 3,2′-dimethylaminobiphenyl, but a considerable number of bladder tumors in Slonaker-strain rats after prolonged injection of the chemical. Intestinal tumors usually appeared much earlier in Wistar than in Slonaker-strain rats. Animals of the latter strain, however, developed a higher incidence of bladder tumors following administration of 3,2′-dimethylaminobiphenyl than did the rats of the Wistar strain. The neoplasms noted in a variety of other organs were essentially alike in the 2 strains.

The induction of tumors at a varied number of sites distant from the portal of entry of a chemical carcinogen greatly complicates the interpretation of the influence of chemical structure as well as other factors on carcinogenesis. However, when compounds are found possessing a high carcinogenic activity for some specific site, organ, or tissue, then studies of the intermediary metabolism of such compounds may be increasingly helpful in elucidating some of the mechanisms of carcinogenesis. Studies of the intermediary metabolism of *N*-2-fluorenylacetamide have yielded

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[2] Presented in part by title before the American Association for Cancer Research, San Francisco, Calif., April 1955.
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[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

evidence of extensive hydroxylation of the benzene ring at positions 5 and 7, but a much smaller amount of hydroxylation has thus far been found to occur at other positions. Furthermore, no experimental evidence has yet been obtained in this laboratory indicating that the methylene bridge is the site of degradation of this compound during its *in vivo* metabolism by the rat (*8*).

This report presents the results obtained from several empirical experiments designed to determine the carcinogenic effect in the rat of compounds lacking the methylene bridge of *N*-2-fluorenylacetamide.

Materials and Methods

Eighteen female Buffalo-strain rats were used in each experiment (*9*). Females were used because it was desired to determine the carcinogenic potentialities of the various chemicals tested for mammary glands. While it was recognized that females may be more resistant then male rats to liver-tumor induction, the males usually are almost completely resistant to mammary-tumor induction, and it was often impossible to test both sexes in adequate numbers with the amount of chemical available.

The drugs were dissolved in propylene glycol and then incorporated into diet #222.[5] This diet of high nutritive quality is composed mostly of natural food stuffs. The chemicals[6] were incorporated at a level of 0.025 percent of the complete diet. Each animal was housed in a separate cage with an individual food cup. Food was allowed *ad libitum*. Food consumption and body weight were recorded for each animal at weekly intervals. Tap drinking water was available to each rat at all times. Any portion of the mixed diet which was not fed to the rats was stored in a refrigerator until used, but storage was usually not over 2 or 3 weeks' duration.

The acetyl derivatives were used because of their greater stability against oxidative decomposition than the free aromatic amines. The use of the acetyl derivative is especially important when the compounds are incorporated into the diet.

The animals were maintained on the drug-containing diet until death appeared imminent. In some cases, when the supply of chemical became exhausted and a large number of apparently healthy animals remained free of gross tumors, they were continued for an additional period of 1 to 3 months on the same basal diet without the drug. Ten rats receiving *N*-2-biphenylacetamide were taken off the chemical after 57 weeks and placed on the basal diet for the duration of the experiment. The last surviving rat of this group was autopsied during the 111th week. The rats receiving *N*-4-biphenylacetamide were continued on the drug throughout the experiment. The rats given *N*-4-(4′-fluoro)biphenylacet-

[5] Diet #222 was composed of the following constituents by weight, in grams: commercial casein 3.00, skim-milk powder 22.75, whole-wheat meal (hard spring) 60.52, brewer's dehydrated yeast 1.00, whole-liver powder 1.00, sodium chloride 1.40, ferric citrate 0.13, cod-liver oil 2.00, corn oil 5.20, and propylene glycol 3.00.

[6] The chemicals used in these experiments were synthesized in the Cancer Research Laboratory (Dr. F. E. Ray, Director), University of Florida, Gainesville, Fla. The m. p. for each compound was as follows: *N*-4-biphenylacetamide 173–174°, *N*-2-biphenylacetamide 120°, *N*-4-(4′-fluoro)biphenylacetamide 205–207°, *N,N′*-diacetylbenzidine 336–338°, and *N,N,N′,N′*-tetramethylbenzidine 195–196°.

amide received the drug for 54 weeks, if they survived that long, and the basal diet thereafter. The last animal was autopsied after 86 weeks. Rats receiving the drugs *N,N'*-diacetylbenzidine and *N,N,N',N'*-tetramethylbenzidine survived for only 2.0 and 3.2 months, respectively, because of the very high toxicity of these 2 compounds (*10*).

All rats were carefully autopsied, except a few animals whose tissues were found to show extreme autolysis. A few animals died of pneumonia too early in the experiment for an adequate test of carcinogenicity and were discarded. Tissues were fixed in Zenker-formol solution and stained with hematoxylin and eosin.

Results

The average daily intake of carcinogen was essentially the same for the rats receiving the 3 carcinogens on which adequate tests were made: *N*-4-, *N*-2-, and *N*-4-(4'-fluoro)biphenylacetamide (table 1). The average daily intake for the first 47 weeks of each of the 3 experiments was 2.3, 2.7, and 2.6 mg. per rat, respectively. When expressed as average daily carcinogen intake in mg. per kg. weight for the same period, the values were 11.3, 11.4, and 13.8, respectively. All the rats receiving *N*-4-biphenylacetamide survived for the first 47 weeks, with an average intake of the chemical for this period of 759 mg. The 3 rats surviving for 70 weeks had an intake of the chemical averaging 1143 mg. (table 1). Animals receiving *N*-2-biphenylacetamide that survived 47 weeks consumed, on the average, 891 mg. of that chemical, and those surviving for 57 weeks averaged 1073 mg. The average total intake of *N*-4-(4'-fluoro)biphenylacetamide for the 47-week period was 858 mg., and the 9 rats surviving for 54 weeks averaged a total of 963 mg. Each rat consumed substantial quantities of one or the other of the 3 substances

TABLE 1.—*Carcinogen intakes for various periods*

Compound	Period (*weeks*)	Number of rats*	Average body weight (*gm.*)	Average daily food† intake per rat (*gm.*)	Average daily carcinogen intake per rat (*mg.*)	Average daily carcinogen intake (*mg./kg.*)	Average cumulative intake per rat (*mg.*)
4-BAA‡.....	1–47	18	204	9. 3	2. 3	11. 3	759
	48–60	12	230	9. 4	2. 4	10. 3	989
	61–70	3	223	8. 6	2. 2	9. 4	1143
2-BAA§.....	1–47	11	232	10. 6	2. 7	11. 4	891
	48–57	10	246	10. 5	2. 6	10. 7	1073
4'F-4BAA‖....	1–47	12	186	10. 3	2. 6	13. 8	858
	48–54	9	212	11. 8	3. 0	13. 9	963

*The number of animals alive during the last week of each period.

†The values in this column are the average daily food intakes for each week of the period. They were calculated from individual food-intake records.

‡4-BAA = *N*-4-biphenylacetamide.

§2-BAA = *N*-2-biphenylacetamide.

‖4'F-4BAA = *N*-4-(4'-fluoro)biphenylacetamide.

under test, and no evidences were noted of toxic effects at the level of drug ingested, although no tests were made to determine the influence of a higher intake on toxicity or carcinogenic potency.

The carcinogenic properties of ingested *N*-4-biphenylacetamide, a compound having the acetylamino group in the same relative position as it is in *N*-2-fluorenylacetamide, is illustrated in table 2. Buffalo-strain female rats developed tumors at only 2 sites—breast and uterus. The breast tumors, verified by microscopic examination, were found in 12 of 17 rats examined. These breast tumors were distributed as follows (table 2): 5 rats without tumors, 8 with 1 each, and 4 with 2 each. Two sarcomas of the uterus developed in rats which were 18 and 20 months of age, respectively, at the time of autopsy (table 2).

Four tumors were found in 11 rats autopsied after ingesting *N*-2-biphenylacetamide for 8 or more months. The acetylamino group of this compound occupies a structural position most nearly corresponding to *N*-4-fluorenylacetamide. One of the 4 tumors was a fibrosarcoma of the breast and the other 3 were tumors of the uterus. One, an undifferentiated uterine carcinoma, developed in a rat 21 months of age, 1 uterine adenocarcinoma occurred in a rat 25 months of age, and the other uterine tumor was an adenocarcinoma and squamous-cell carcinoma found in a 31-month-old rat. Uterine tumors are quite rare in Buffalo-strain rats of comparable or older ages and when they do appear they usually are sarcomas (Dr. K. E. Snell, NCI, personal communication). The carcinogenicity of *N*-2-biphenylacetamide as indicated in these experiments is low compared to that of *N*-4-biphenylacetamide, but the uterine tissue appears to have a slight susceptibility to the induction of tumors by the former. The *N*-4-fluorenylacetamide has slight, if any, carcinogenicity as found in Buffalo-strain female rats during a preliminary test in this laboratory (*11*). Therefore, the carcinogenicity of *N*-4-fluorenylacetamide more nearly corresponds to that of the structurally comparable *N*-2-biphenylacetamide.

The incorporation of the halogen fluorine in the 4′ position of *N*-4-biphenylacetamide to give *N*-4-(4′-fluoro)biphenylacetamide, greatly increases the carcinogenic properties of this biphenyl derivative as shown in table 2. The tissues of 16 rats disclosed numerous distant tumors induced at 7 different sites. The largest number of tumors noted were carcinomas of the breast, but a few were carcinosarcomas. Breast-tumor frequency per rat is given in the footnotes in table 2. Only 1 rat of the 16 examined microscopically failed to show a breast tumor. Two animals had single breast tumors, 7 had 2 each, 4 had 3 each, and 2 had 5 each, making a total of 38 tumors. The liver was the site where the next largest number of tumors occurred, with hepatomas present in 11 animals. Seven animals had epithelial neoplasms of the kidney. Some kidney tumors were multiple, and in 1 animal bilateral kidney tumors occurred. Two acoustic-duct tumors, 1 uterine tumor, 1 tumor of the small bowel, and 1 tumor of the spleen were also found in this group.

The sarcoma of the spleen arose in a rat 10 months after the start of

TABLE 2.—*Carcinogenic properties of certain ingested derivatives of mono- and diaminobiphenyl compounds*

Compound	Number of rats / Number of rats with tumors	Average age at autopsy (*months*)	Average length drug feeding (*months*)	Site of tumor						
				Breast	Kidney	Ear	Uterus	Small bowel	Liver	Spleen
N-4-biphenylacetamide	17/13	16. 8	13. 0	16*	0	0	2†	0	0	
N-2-biphenylacetamide	11/4	20. 0	15. 0	1‡	0	0	3§	0	0	
N-4-(4′-fluoro)biphenylacetamide	16/16	14. 3	11. 7	38‖	8¶	2**	1††	1‡‡	11	1§§
N,N′-diacetylbenzidine	15/1	6. 0	2. 0	0	0	1‖‖	0	0	0	
N,N,N′,N′-tetramethylbenzidine	9/0	6. 8	3. 2	0	0	0	0	0	0	

*Fifteen adenocarcinomas, 1 squamous-cell carcinoma.
†Two sarcomas in rats 18 and 20 months of age at autopsy.
‡One fibrosarcoma in a rat 18 months of age at autopsy.
§One adenocarcinoma in a rat 25 months old at autopsy. One undifferentiated carcinoma in a rat 21 months old at autopsy. One adenocarcinoma and squamous-cell carcinoma in a rat 31 months old at autopsy.
‖Frequency of breast tumors: 0 in 1 rat, 1 in 2 rats, 2 in 7 rats, 3 in 4 rats, 5 in 2 rats; 35 carcinomas, 3 carcinosarcomas.
¶Epithelial neoplasms: single, bilateral, and multiple; bilateral counted as separate tumors; multiple tumors counted only once.
**Squamous-cell carcinomas.
††Sarcoma.
‡‡Mucoid carcinoma.
§§Sarcoma.
‖‖Drug fed to this rat for 2 months, withheld for 8 months; then drug fed on alternate weeks for next 9 weeks. Death at 14 months.

the experiment and, since it was the only tumor found in this animal, we believe it was probably of spontaneous origin. This view is supported by the absence of primary tumors of the spleen in later experiments, where this same compound was ingested by both male and female Buffalo-strain rats.

The 2 diaminobiphenyl compounds tested, *N,N*-diacetylbenzidine and *N,N,N′,N′*-tetramethylbenzidine, were extremely toxic. The average length of time these compounds were fed was 2.0 and 3.2 months, respectively. Both were without carcinogenic activity during this brief interval (table 2). That *N,N*-diacetylbenzidine may be slightly carcinogenic is suggested by the development of a single squamous-cell carcinoma of the ear duct in a rat fed the drug continuously for 2 months, followed by a drug-free period of 8 months, then a 9-week terminal period during which the drug was incorporated in the diet during alternate weekly intervals. The ear tumor was present in this 14-month-old rat at autopsy. Ear tumors have not been observed in this laboratory in Buffalo-strain female rats which were on a stock diet or a control diet.

Microscopic Pathology

N-*2-biphenylacetamide adenocarcinoma of the uterus.*—The entire uterine cavity was replaced by a neoplastic glandular tumor with papillary features and with extension into the myometrium proper. The tumor glands were separated by a dense *fibromuscular-like* stroma. In some areas within the myometrium, small cords of neoplastic cells were present. In these areas the stroma was more fibrous and closely resembled the so-called "scirrous" adenocarcinoma of the breast. The nuclei were quite pleomorphic and vesicular, and possessed nucleoli. Mitoses were common. The cytoplasm was finely granular and varied considerably in the amount present (fig. 1).

N-*4-biphenylacetamide fibrosarcoma of the uterus.*—The cellular density of the tumor varied from areas with only a few nuclei to others where only a sparse amount of interstitial stroma was apparent, due to the numerous closely associated nuclei (fig. 2). The matrix background was loose, with a faint fibrillar structure. The nuclei were quite pleomorphic; however, the basic cell was bluntly spindle-shaped with the long axes parallel one to the other, thus forming broad, intertwining cords of cells. No distinct cell walls were apparent with hematoxylin and eosin. A moderate number of large giant cells and other nuclei of various sizes and shapes were scattered between the pleomorphic nuclei. Nucleoli were present in most of the cells. Mitoses were minimal. Throughout the tumor a few small vascular spaces were apparent.

N-*4-(4′-fluoro) biphenylacetamide adenocarcinoma of the breast.*—The tumor was composed of large masses of neoplastic cells separated one from the other by broad bands of moderately cellular fibrous stroma (fig. 3). Within these individual, well-demarcated neoplastic growths the tumor had a glandular appearance, with some areas showing a papillary arrange-

ment as well. The blunt papillary fronds and glandular elements were separated one from the other by varied amounts of fibrous connective-tissue stroma varying from broad to delicate strands. Within this interstitial stroma individual and small clumps of neoplastic cells were proliferating. A few vascular spaces were present. Under higher magnification the nuclei were seen to be quite uniformly round and vesicular and to possess nucleoli. Mitotic figures were quite numerous. An eosinophilic, homogeneous substance was present within many of the neoplastic glands. Small focal areas of necrosis were scattered throughout.

N-4-(4'-*fluoro*)*biphenylacetamide hepatoma with lung metastases.*—Histologically the tumor was sharply delineated from the adjacent normal liver parenchyma. At the junction of the tumor and normal liver the latter cells were not abnormally compressed, thus giving the impression that the tumor was not growing from the margins but rather that the parenchymal cells had changed to neoplastic elements and had then become joined to the neoplastic growth (fig. 4). The tumor was composed of recognizable liver cells; however, their nuclei were larger and more vesicular and contained prominent nucleoli. Mitoses were rare. The cytoplasm of the cells was finely granular and distinctly darker in staining quality than the adjacent normal parenchymal cells. The tumor cells formed cords composed of several layers of cells, and the cords were separated by sinus spaces that appeared to be lined by both endothelial and Kupfer cells.

This tumor metastasized to the lung (figs. 5 and 7), where it possessed almost the same histologic features as described above, except that the sinus spaces were less clearly defined.

N-4-(4'-*fluoro*)*biphenylacetamide hepatoma with cholangiolar component, bile-duct ectasia, cystic degeneration, or cystic features.*—In this section the neoplastic cells were associated with a well-defined, irregular, cystically dilated glandular or tube-like element (fig. 6). The epithelium lining these cystic elements was of a plump cuboidal character. The nuclei were round and vesicular and possessed small nucleoli. Occasionally there was some nuclear reduplication. The contents of the lumen varied from emptiness to an amorphous granular nonspecific material containing red blood cells and an occasional white blood cell. A few scattered vascular spaces were present between the cystic spaces and liver tumor cells. Although the liver tumor cells were essentially the same as those shown in figure 4, there seemed to be a less orderly arrangement of the cords, with consequent blurring of the sinus spaces.

There were no metastases noted in the lung with the above cystically dilated glandular or tubular elements, although small tubular structures lined by plump cuboidal epithelium could be identified.

N-4-(4'-*fluoro*)*biphenylacetamide epithelial kidney tumor.*—Two of the largest tumors arising in the kidneys, in this case from the same rat, are illustrated in figure 8. These tumors were much lighter in color than the kidney, although the gross photograph shows extensive vascular supply to the tumor. A microscopic section of an entire tumor in the kidney of the rat (fig. 9) well illustrates one of the typical epithelial neo-

plasms of the kidney observed in the rats after ingesting *N*-4-(4′-fluoro)-biphenylacetamide.

Discussion

The elimination of the methylene bridge of *N*-2-fluorenylacetamide, as in the comparable biphenyl compound, *N*-4-biphenylacetamide, definitely decreased carcinogenicity of this aromatic amine in the liver, noted by Miller *et al.* (*3,5*), by Morris (*1*), and by Walpole *et al.* (*6*) in the related 4-aminobiphenyl. The presence of the methylene bridge, however, is not essential for liver-tumor development since *N*-4-(4′-fluoro)biphenylacetamide, the aminobiphenyl compound containing the halogen fluorine in the 4′ position, induced a high incidence of liver tumors in female rats (*1,2*). An equally high incidence of liver tumors has been noted by Miller *et al.* (*5*) in Sprague-Dawley male rats after ingestion of a diet containing *N*-4-(4′-fluoro)biphenylacetamide for 12 months. Their dose of the chemical was 50 percent higher than that used in the experiments reported here with Buffalo-strain female rats, but the length of the feeding period was about the same. It is not yet known whether there is a sex difference in the induction of liver tumors by *N*-4-(4′-fluoro)biphenylacetamide. Male Buffalo-strain rats ingesting *N*-4-(4′-fluoro)biphenylacetamide, in uncompleted experiments, appear to be showing about the same incidence of liver tumors as that found for the females. The *N*-4-dimethylaminobiphenyl compound apparently also has a slight carcinogenic activity on the liver, for Miller *et al.* (*4*) noted 3 benign hepatomas after 10 months in male rats that had ingested the chemical for 8 months.

N-4-(4′-fluoro)biphenylacetamide is considered by Miller and Miller (*5*) to be a weak hepatocarcinogen, although the data used to support this view shows that 5 of 8 male Sprague-Dawley rats had developed liver tumors in 12 months. The rats ingested the chemical during a 10-month-period, whereas 12 of 16 male rats that ingested *N*-4-(7-fluoro)fluorenylacetamide for 3 months developed liver tumors by 6 months. Such data do not uphold strongly the interpretation that a significantly higher incidence of liver tumors develop in the *N*-2-(7-fluoro)fluorenylacetamide than in the *N*-4-(4′-fluoro)biphenylacetamide group, although the tumors did appear earlier and with a lower dose of carcinogen. The incidence of liver tumors (11 of 16 female rats) in the group ingesting *N*-4-(4′-fluoro) biphenylacetamide (table 2) suggests this compound to be a potent hepatocarcinogen at approximately the same dietary level used by the Millers (*5*), 0.020 percent *versus* 0.025 percent in our study. The data (table 2) further suggest that the female rat is highly susceptible to liver-tumor induction by *N*-4-(4′-fluoro)biphenylacetamide.

This report is the first, to our knowledge, describing a high incidence of renal epithelial neoplasms in rats after ingesting *N*-4-(4′-fluoro)biphenylacetamide. Wilson *et al.* (*12*) observed 1 carcinoma of the renal pelvis in the kidney of a rat that had ingested *N*-2-fluorenylacetamide. They also noted frequent hyperplasia of the kidney pelvis. Miller *et al.* (*5*) made no mention of kidney tumors in male Sprague-Dawley rats ingesting *N*-4-(4′-fluoro)biphenylacetamide, but Hendry *et al.* (*13*), in a preliminary

report, noted tubular kidney tumors in male Wistar-strain rats injected with *N*-4-(4′-fluoro)aminobiphenyl. Eker (*14*) has reported familial renal tubular adenomas in Wistar rats at the relatively early age of less than 6 months, which suggests that their development stemmed from embryonic derangement. The tumors noted by Eker were usually multiple and bilateral.

The kidney tumors observed in this laboratory developed in rats fed the diet containing *N*-4-(4′-fluoro)biphenylacetamide for about 1 year (average 11.7 months). The animals had attained an average age of 14.3 months. Nuclear atypia of the cells of the renal tubule was first noted in animals that had ingested the chemical for 9½ months, although the first renal neoplasm was not observed until 10½ months. Microscopic changes may have occurred somewhat earlier and escaped detection because all portions of the kidney had not been examined prior to the discovery of the first neoplasm. Additional experiments designed to study the histogenesis of these tumors have been carried out, and the results will be reported in a subsequent paper.

The absence of intestinal tumors in the rats ingesting acetylated aminobiphenyl compounds and the presence of a high incidence of intestinal tumors after subcutaneous administration or ingestion of 4-aminobiphenyl by Walpole *et al.* (*6*) poses the question of whether the strain of rat, the route of administration, or other factors have a significant role in the site specificity and carcinogenic potency of the aminobiphenyls. One would expect the free aminobiphenyl to be formed readily *in vivo* in rats ingesting *N*-4-biphenylacetamide. On the other hand, 4-aminobiphenyl may be more readily oxidized *in vivo* to a more active carcinogen. The concentration in the organism may be greatly different, too, depending on the dose, the route of administration, and the vehicle employed. These are important questions which studies of the intermediary metabolism of aromatic aminobiphenyls may be expected to resolve in future investigations.

Summary and Conclusions

Substantial amounts of *N*-2- , *N*-4- , and *N*-4-(4′-fluoro)biphenylacetamide were ingested at a 0.025 percent dietary level by Buffalo-strain female rats during experimental periods of 12 to 15 months. *N,N,N′,N′*-tetramethylbenzidine and *N,N′*-diacetylbenzidine, when ingested at the same level, were exceedingly toxic to the rats. Due to this high toxicity the average survival periods were only 3.2 and 2.0 months, respectively. Distant tumors were induced at one or more sites by each of the monoacetylaminobiphenyl compounds, no tumors were induced by tetramethylbenzidine, and an ear-duct tumor developed in 1 rat that received diacetylbenzidine intermittently during a 14-month period. Three uterine carcinomas developed in 11 rats ingesting *N*-2-biphenylacetamide for 8 or more months, indicating that uterine tissue is somewhat susceptible to tumor induction by this compound.

Fifteen adenocarcinomas and 1 squamous-cell carcinoma of the breast were found in 12 of 17 animals after ingestion of *N*-4-biphenylacetamide.

Two uterine sarcomas, probably of spontaneous origin, were also found in two 18- and 20-month-old rats in this experiment. No liver tumors developed in the *N*-2- and *N*-4-biphenylacetamide experiments.

N-4-(4′-fluoro)biphenylacetamide induced tumors at 7 sites in 16 rats. Thirty-five carcinomas, and 3 carcinosarcomas of the breast were noted in 15 rats. Only 1 rat of the group failed to develop at least 1 breast tumor. Eleven of the 16 developed hepatomas, while 7 developed epithelial neoplasms of the kidney. The kidney tumors were often multiple and in 1 animal they were bilateral. Moreover, the animals in this experiment developed 2 squamous-cell carcinomas of the ear duct, and 1 tumor each of the uterus, small bowel, and spleen. *N*-4-biphenylacetamide produced no liver tumors in these experiments. This compound has a chemical structure similar to the potent hepatocarcinogen *N*-2-fluorenylacetamide minus the methylene bridge at the 9-position of the fluorene ring. The incorporation of the halogen fluorene into the 4′ position of *N*-4-biphenylacetamide converts *N*-4-biphenylacetamide, which is inactive for liver, not only into a potent hepatocarcinogen, but increases its carcinogenic activity for the rat mammary gland, kidney, and numerous other sites.

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PLATE 10

FIGURE 1.—Adenocarcinoma of uterus in rat after ingestion of *N*-2-biphenylacetamide; uterine cavity at *upper left*, tumor in *center*, myometrium on *right*. × 100

FIGURE 2.—Fibrosarcoma of uterus in rat after ingestion of *N*-4-biphenylacetamide; uterus at *upper left*, tumor *below* and to the *right*. × 250

FIGURE 3.—Adenocarcinoma of breast in a female rat after ingestion of *N*-4-(4′-fluoro)-biphenylacetamide. × 100

FIGURE 4.—Hepatoma in a female rat after ingestion of *N*-4-(4′-fluoro)biphenylacetamide. Hepatoma on *left*. *Note* normal liver cells on *right* are not abnormally compressed. × 100

FIGURE 5.—Hepatoma metastatic to lung of rat; primary hepatoma shown in figure 4. This metastatic lesion has histologic features almost identical with hepatoma illustrated in figure 4. × 100

FIGURE 6.—Hepatoma in female rat with cholangiolar component and with cystic features after ingestion of *N*-4-(4′-fluoro)biphenylacetamide. No lung metastases of this tumor type were observed. × 100

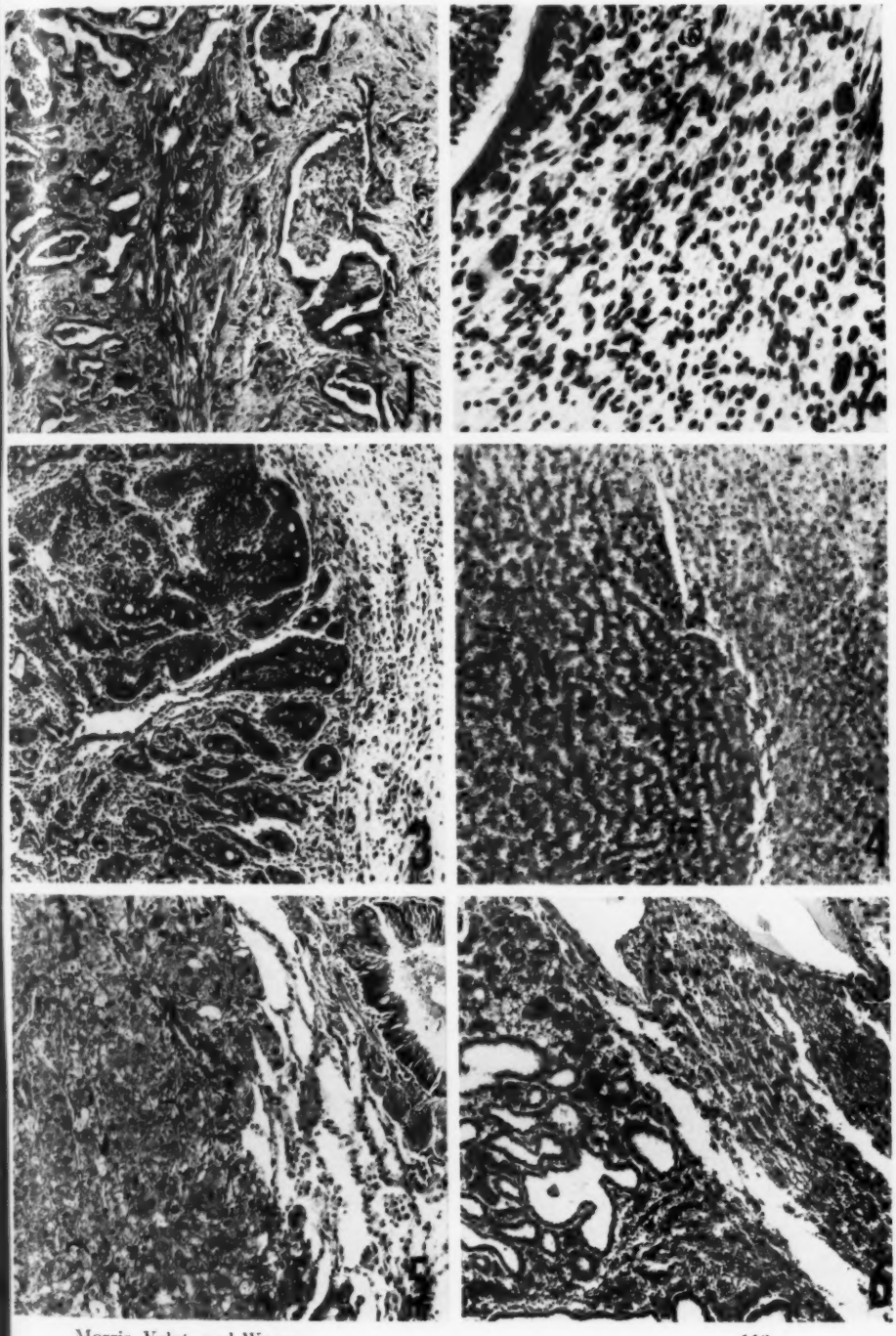

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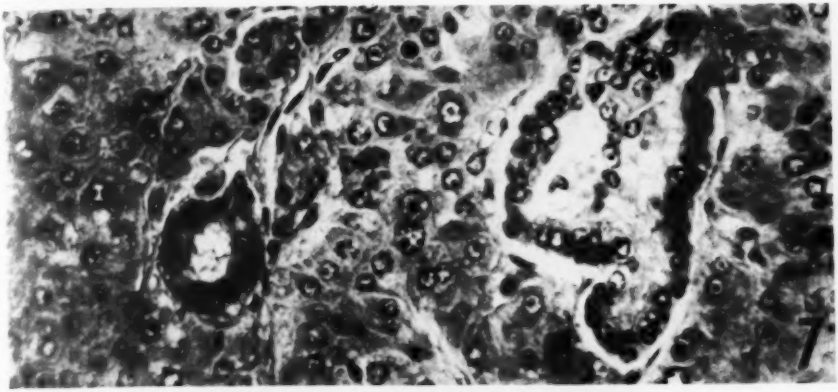
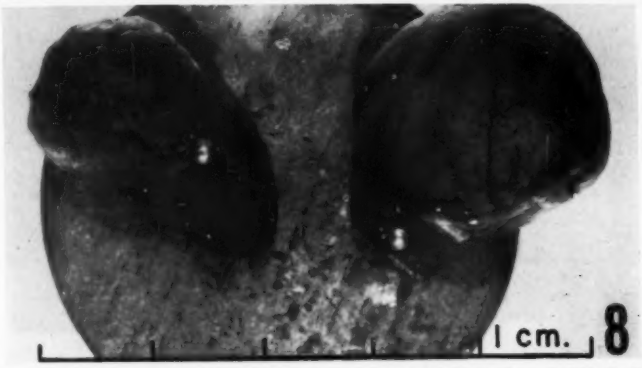
PLATE 11

Figure 7.—Bile ducts in a hepatoma metastatic to lung of rat. The primary hepatoma is shown in figure 4. × 380

FIGURE 8.—Bilateral kidney tumors in rat that ingested *N*-4-(4′-fluoro)biphenylacetamide. *Note* light-colored tumors extending out from the exterior surface of kidney. The extensive vascular system can be noted on surface of tumors. Portions of normal-appearing kidney may be seen below the tumors.

FIGURE 9.—Section of an entire epithelial tumor of the kidney in a rat that ingested *N*-4-(4′-fluoro) biphenylacetamide. Normal kidney tissue in *upper left* portion of photomicrograph. × 200

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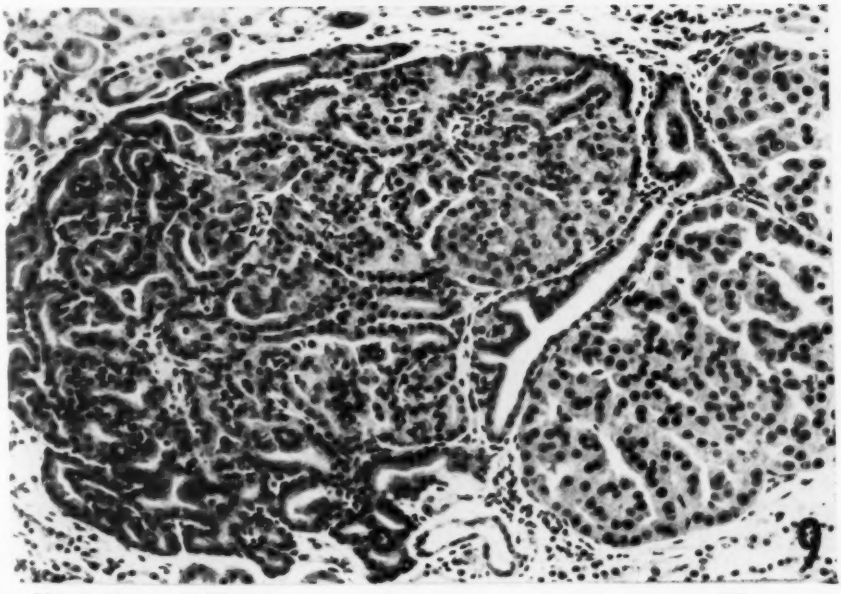

Comparison of the Reduction of Two Tetrazolium Salts with Succinoxidase Activity of Tissue Homogenates[1]

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During the course of some experiments on the chemical induction of liver tumors in mice, it was observed that frozen sections of small primary liver tumors had a greater capacity to reduce neotetrazolium histochemically than did the cells of the surrounding, nontumorous liver (*1*). Some years previously, it had been shown that the manometrically determined succinoxidase activity of azo dye-induced rat-liver tumors and of a transplantable spontaneous mouse-liver tumor was *less* than that of normal liver (*2,3*). The discrepancy in these findings raised the question as to whether tetrazolium reduction by tissues, either as frozen sections or as homogenates, was a valid measure of succinic-dehydrogenase activity. The experiments reported here were designed, first, to test whether the reduction of tetrazolium salts by tissue homogenates satisfied some of the criteria for measuring enzyme activity and, second, to compare tetrazolium reduction with oxygen uptake as a measure of succinic-dehydrogenase activity in various tissues.

Method

Ten percent water homogenates of liver, kidney, and brain from strain A mice of both sexes were used as a source of enzyme. In experiments with frozen tissue, the homogenate was divided and one half of it was frozen immediately in an alcohol-dry ice mixture. After 5 to 10 minutes the homogenate was slowly thawed by warming in the hand and was thoroughly mixed but not re-homogenized before use. In the preparation of the incubation medium the following materials were used: neotetrazolium chloride (NT), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), sodium succinate (0.5 *M*), tris-hydroxymethylaminomethane (0.2 *M*), potassium cyanide (2×10^{-3} *M*), calcium chloride (4×10^{-3} *M*), and aluminum chloride (4×10^{-3} *M*).

Incubation was carried out in 25 ml. Erlenmeyer flasks shaken in a constant-temperature water bath at 37.5° C. The reaction was stopped

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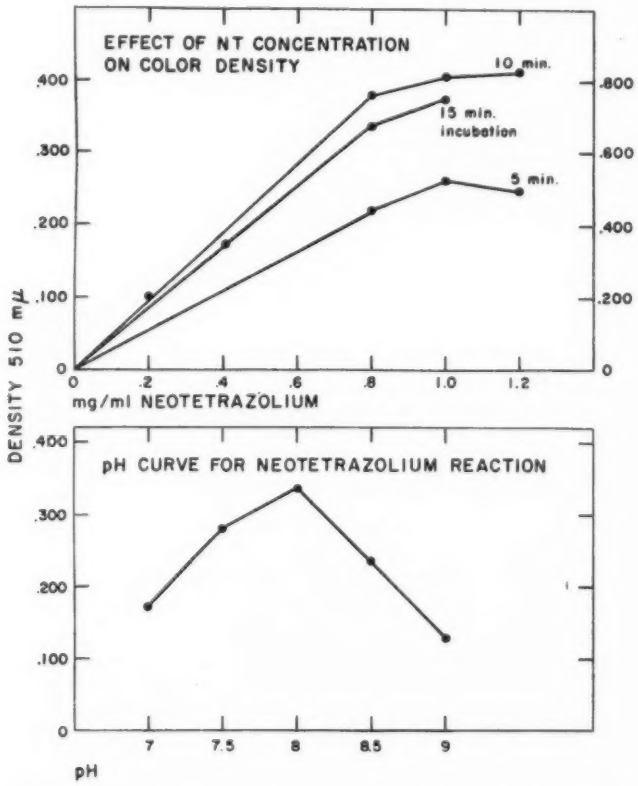
by the addition of 0.5 ml. of 40 percent trichloroacetic acid; 3 ml. ethyl acetate were added; the flasks were stoppered with silicone rubber stoppers and shaken 50 times to extract the formazan. Extraction of the formazan from the TCA-substrate-tissue mixture was complete. After shaking, the contents of the flasks were decanted into 12 ml. centrifuge tubes and centrifuged 2 minutes at 2000 r.p.m. to clear the ethyl acetate extract of tissue precipitate. The density of the color was read at 510 mμ for NT formazan and 490 mμ for INT formazan on a Beckman model DU spectrophotometer, using 1 cm. cuvettes. Since some color developed in the absence of added succinate in this reaction, the color in the experimental flasks was corrected by subtracting blanks, which consisted of all components of the substrate mixture except succinate. All determinations were run in duplicate.

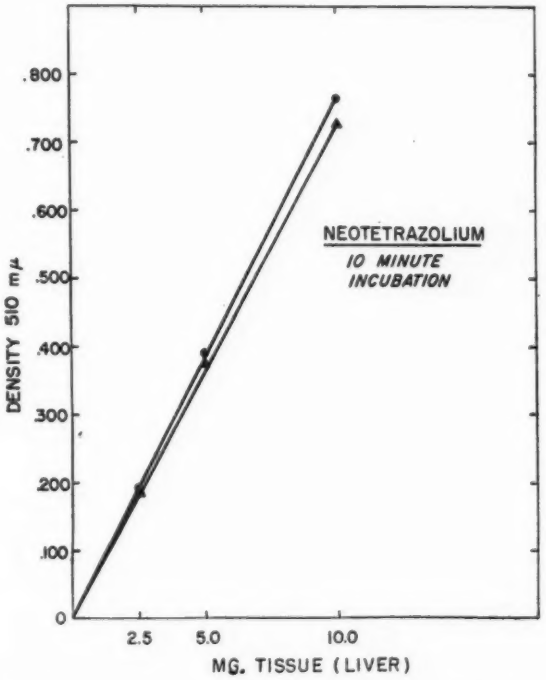
The manometric determinations of succinoxidase activity were performed according to the method of Schneider and Potter (*4*).



TEXT-FIGURE 1.—Effect of *p*H and NT concentration on color density. In *top* graph, read scale at right for 15-minute incubation. In *lower* graph, NT concentration was 1 percent and incubation time was 10 minutes.

Results

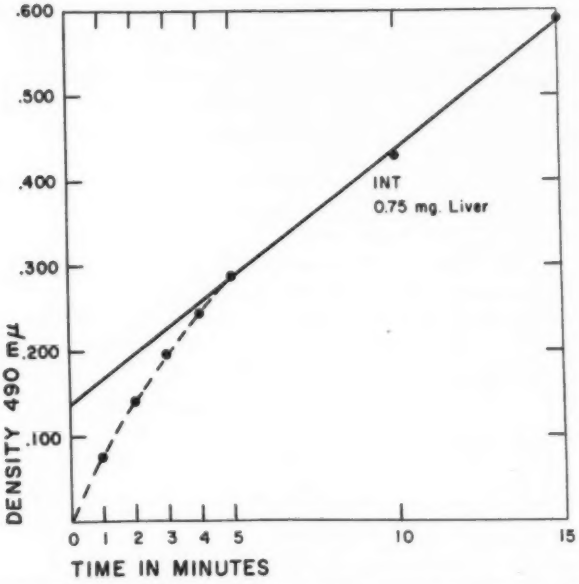
Mouse-liver homogenates were used in working out a reproducible technique for measuring the enzymatic reduction of the tetrazolium salts in the presence of succinate. It was found that maximal color development depended upon both tetrazolium concentration and *p*H (text-fig. 1).



TEXT-FIGURE 2.—Tissue-concentration curve for NT incubated 10 minutes at 37.5° C. *Circles* represent frozen-thawed homogenate; *triangles* represent untreated homogenate of mouse liver.

The addition of calcium and aluminum ions to the substrate mixture did not appreciably increase the reduction of tetrazolium, although there was an indication of slight but variable stimulation in the presence of cyanide. This confirmed the findings of Jardetzky and Glick (*5*).

The rate of reduction of tetrazolium by liver homogenates was linear with increasing amounts of enzyme, and freezing and thawing the homogenates had no appreciable effect upon this rate (text-fig. 2). When enzyme (tissue homogenate) was added to the incubation mixture, the rate of reduction of tetrazolium was linear with time between 5 and 20 minutes for NT and between 5 and 15 minutes for INT (text-fig. 3). It can be seen that the curve does not pass through the origin. The behavior of the time-density curve was clarified when tissue was incubated with INT for intervals of 1 to 5 minutes (text-fig. 3). Between 0 and 5 minutes



TEXT-FIGURE 3.—Time-density curve. *Dotted line* represents non-linear rate of reduction of INT between 0 and 5 minutes.

of incubation INT formazan is produced at a non-linear rate (*dotted line* on graph), while between 5 and 15 minutes, the rate of tetrazolium reduction proceeds in a straight line.

These results formed the basis for a standard procedure that was used for comparison of tetrazolium reduction with oxygen uptake. Tissues in varying amounts were incubated in a medium consisting of 1 ml. buffer, 0.25 ml. sodium succinate, 0.25 ml. NT (1%), and distilled water to make 2.5 ml. When INT was used, much smaller amounts of tissue were needed, so that the volume of the incubation medium was reduced to 0.5 ml. buffer, 0.1 ml. succinate, 0.1 ml. INT (1 %), and water to make 1 ml.

Comparison of tetrazolium reduction with oxygen uptake.—One way to test the validity of the tetrazolium method was to compare the ratios of the enzyme activities of several tissues measured by oxygen uptake with the ratios obtained by measuring tetrazolium reduction. If similar ratios were found, it would be a good indication that, on a relative basis, the tetrazolium method measured the enzyme system with the same adequacy as did the manometric method. Table 1 summarizes the results of oxygen uptake and tetrazolium-reduction measurements, using homogenates of liver, kidney, and brain. It can be seen that there is a very close correspondence between the ratios of the succinic-dehydrogenase activity of these 3 tissues when determined by either method.

In addition to a relative comparison of the 2 methods, further information was sought concerning the efficiency of the tetrazolium method as

TABLE 1.—*Oxygen uptake and neotetrazolium reduction by normal mouse tissues**

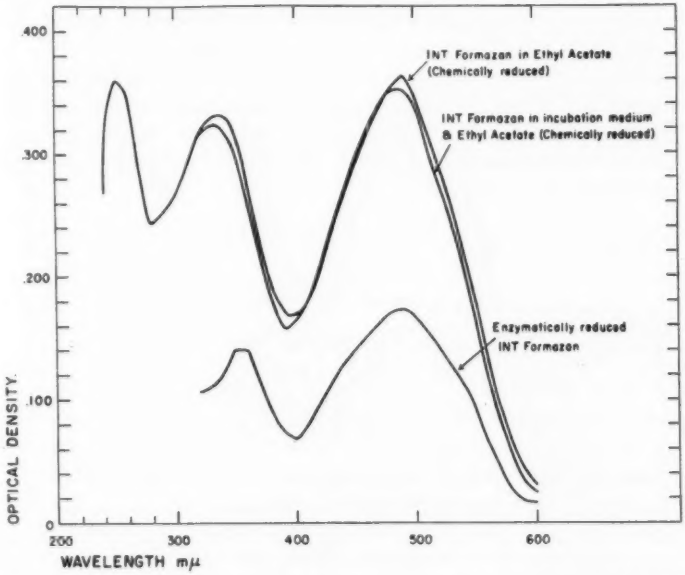
Tissue	O_2 ($\mu l.$)	Ratio	Optical density	Ratio
Liver	55.0 (11)	1. 00	0.786 (12)	1. 00
Kidney	96.4 (4)	1. 75	1.303 (5)	1. 65
Brain	17.6 (3)	0. 32	0.259 (8)	0. 33

*Per 10 mg. tissue per 10 min. at 37.5° C.

The numbers in parentheses indicate the number of mice used to calculate the averages given. "Ratio" signifies the relative activities of liver, kidney, and brain with respect to liver.

compared to the manometric method by determining the number of moles of formazan reduced and the amount of oxygen consumed by a given amount of tissue during a specified time interval.

In order to calculate the molar extinction of the reduced tetrazoles, formazans of NT and INT were prepared by reduction with sodium hydrosulfite. An additional NT formazan was prepared by reduction with dextrose at *p*H 12 (*6*). Standard solutions were prepared by dissolving weighed amounts of the formazans in 100 ml. ethyl acetate. The absorption curves of both chemically and enzymatically reduced INT had a peak at 490 mμ (text-fig. 4). Additional peaks were observed at lower wave lengths, but these were obscured in the enzymatically produced formazan



TEXT-FIGURE 4.—Absorption curves for formazans of INT produced by chemical and enzymatic reduction. Most of the readings were made at 10 mμ intervals; the peaks were measured at 2 mμ intervals. The formazan solution used for the middle curve was layered over the complete incubation medium plus tissue, mixed by shaking, then centrifuged, and read.

by the presence of trichloroacetic acid in the medium. The absorption curves of the NT formazan produced by hydrosulfite and dextrose reduction had similar shapes and broad but reproducible peaks at 540 mμ, whereas the peak absorption of the enzymatically reduced NT occurred at 510 mμ. Because of the shift in position of the absorption peak in the chemically reduced NT formazan, it was not possible to calculate accurately the number of moles of NT formazan reduced enzymatically. Thus, the figures presented below represent merely an order of magnitude with which the INT values may be compared.

When an absorption curve was run on a 5.8×10^{-5} *M* solution of NT formazan, the optical density at 510 mμ was 0.613 and at 540 mμ was 0.659. With a 6.7×10^{-5} *M* solution of INT formazan, the optical density at 490 mμ was 0.360. Since the formazans obey the Beer-Lambert law, their concentration in solution can be termined by the formula:

$$D = KCL$$

where *D* is the optical density, *C* is the molar concentration of the substance in solution, *L* is the length of the light path in centimeters, and *K* is a constant. Using 1 cm. cuvettes, the constants obtained for NT formazan at the 2 wave lengths were 10.6 and 11.2, respectively, while for INT a *K* of 5.34 was obtained. With these constants it was possible to estimate the number of micromoles of formazan produced enzymatically (*C*) and to compare this with the number of micromoles of oxygen consumed by the same amount of tissue during a given time interval.

In comparing the reduction of NT and INT relative to molecular oxygen, it should be noted that NT is a bis-tetrazolium salt requiring 4 electrons for its reduction, while the single tetrazole ring of INT is reduced by 2 electrons (*6*). Comparisons were made on the basis of 10 mg. of tissue incubated for 10 minutes.

Oxygen uptake by liver homogenates (average of 11 mice)
=55.0 μ*l*.
=2.45 μ*M* oxygen

Reduction of NT by liver homogenates (average of 12 mice)
Optical density=0.786 at 510 mμ
=0.074 μ*M* formazan
⇨0.074 μ*M* oxygen

Reduction of INT by liver homogenates (average of 6 mice)
Optical density=6.840 at 490 mμ
=1.28 μ*M* formazan
⇨0.64 μ*M* oxygen

These results show that INT is roughly 10 times more efficient an electron acceptor for measuring succinic-dehydrogenase activity than is NT. Although INT is only one quarter as efficient an electron acceptor as molecular oxygen, it nevertheless has a high efficiency in comparison with

another dye, methylene blue, which is commonly used for measuring this same system (*7*).

Discussion

The purpose of the work presented here was to examine critically, with respect to its validity, a method for the quantitative estimation of the succinic-dehydrogenase system by means of the reduction of tetrazolium salts. In addition, the efficiency of NT and INT as electron acceptors in this system was compared with that of molecular oxygen, which is the final electron acceptor when succinic dehydrogenase is the limiting factor in the whole succinoxidase system. The term "succinic-dehydrogenase system" was suggested by Singer (*8*), who has shown that purified succinic dehydrogenase will not react with methylene blue, indophenol, or the tetrazolium salts. Thus the tetrazolium salts probably do not measure succinic dehydrogenase directly but accept electrons from a chain of electron carriers which have yet to be precisely characterized (*8*). In comparison with NT and methylene blue, INT as an electron carrier has an efficiency only slightly less than that of molecular oxygen. This is a most desirable situation, since it permits a measurement of the activity of the succinic-dehydrogenase system in very small amounts of tissue, such as the small primary tumors which appear in azo dye-fed mice. The efficiency of INT as an electron carrier will facilitate comparison of the enzyme activity of the small tumors with that of small masses of the surrounding, nontumorous liver. It will also allow similar estimates to be made in small organs or in parts of larger, more complex organs. Already, Farber and co-workers (*9*) have made an interesting comparison between manometric measurements and tetrazolium reduction in the kidney. Homogenates of the outer region of the cortex, the proximal portion of the medulla, and the papilla of the kidney gave relative activities, measured manometrically, of 10, 8, and 0.8. These values corresponded closely to the intensity of coloration of these regions in kidney slices incubated with tetrazolium salts.

Some of the data reported here serve to confirm the work of Defendi and Pearson (*10*), Jardetzky and Glick (*5*), and Glick and Nayyar (*11*). Although the methods reported in each case are different in many respects, the results indicate that it is possible to measure the succinic-dehydrogenase system with considerable accuracy in tissue homogenates or slices by using INT. It is doubtful that good "absolute" quantitation with neotetrazolium is possible until further work is done. The discrepancy between the positions of the absorption peaks of the chemically and enzymatically reduced NT indicates that different proportions of the red (mono) and blue (di) reduced formazans are present in each. The fact that neotetrazolium may be reduced to a mono- or di-formazan, each with a different absorption spectrum, means that, with the information currently available, quantitation of mixtures of these compounds present after enzymatic or chemical reduction of the original salt is not possible. As far as the quantitation of the succinic-dehydrogenase system in tissue homogenates is concerned, INT lends itself admirably to this purpose

because only a single electron pair is required for its reduction. Both the present work and that of Defendi and Pearson (*10*) and Glick and Nayyar (*11*) have shown that the absorption spectra of the chemically and enzymatically produced formazan of INT have the same absorption peak, and thus the amount of formazan produced enzymatically can be accurately determined.

Although NT is apparently not a suitable electron acceptor on theoretical grounds, a paradox presents itself in the fact that the amounts of NT formazan produced by homogenates of liver, kidney, and brain are in the same ratio as the amount of oxygen consumed by these tissues. This suggests that when NT is reduced enzymatically, constant amounts of the mono- and di-formazan are produced, and that although it may not be possible to determine the exact amount of the formazan present, a relative comparison of different amounts is possible. However, the fact that INT, in addition to being readily quantitated, is at least 10 times more sensitive than NT in measuring enzyme activity makes it the tetrazolium salt of choice in measuring the succinic-dehydrogenase system in tissue homogenates.

Summary

A method for measuring the succinic-dehydrogenase system in homogenates of mouse liver, kidney, and brain by the reduction of neotetrazolium chloride (NT) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was compared with the manometric method of Schneider and Potter (*4*) for measuring the succinoxidase system. It was found that:

1) The ratio of oxygen consumption of liver, kidney, and brain was 1:1.75:0.32 and the ratio of the optical density of the NT formazan produced by these tissues was 1:1.65:0.33. This indicates that, on a relative basis, the tetrazolium method measures the succinic-dehydrogenase system with the same adequacy as the manometric method.

2) A given amount of liver homogenate in a given time interval will take up 2.45 μM of oxygen, and will reduce INT and NT to produce 1.28 μM and 0.074 μM of formazan, respectively. Thus INT is one quarter as efficient as molecular oxygen, but is roughly 10 times more efficient than NT as an electron acceptor in this system.

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The Distribution and *in Situ* Growth Pattern of Injected Rat Marrow in X-Irradiated Mice [1, 2]

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The modification of acute radiation injury in lethally irradiated mice by the injection of suspensions of isologous or heterologous (rat) bone marrow has now been demonstrated by a number of workers (*1–4*). Recently, a histochemical technique has been used to elucidate the mechanism of the protective action of heterologous marrow (*5*). Since rat leukocytes contain large quantities of alkaline phosphatase and mouse leukocytes relatively little (*6*), blood and bone-marrow smears treated for this enzyme reveal rat cells as black or dark brown, while mouse cells remain unstained. Using this method, it was possible to identify rat marrow injected into irradiated mice, and it could be shown that the rat cells actually repopulated the marrow cavity and, by functioning hematopoietically in the mouse, prevented acute radiation death. The validity of these conclusions has been supported by the work of Lindsey *et al.* (*7*). These workers injected irradiated rats with marrow from rats of a different blood group and subsequently identified red cells of the donor's type circulating in the recipient. Furthermore, Ford *et al.* (*8*) have recently used chromosome markers to identify injected heterologous and homologous marrow in the marrow cavities of irradiated recipient mice, and Makinodan (*9*) has also demonstrated rat cells circulating in lethally irradiated mice after protection with rat bone marrow. Makinodan employed a quantitative immunohematologic technique to identify the rat erythrocytes and the phosphatase method to demonstrate the granulocytes.

The present paper extends the alkaline-phosphatase technique to the study of the actual distribution and growth pattern of rat cells within various organs of the irradiated mouse after either intravenous or intraperitoneal injection. By treatment of tissue sections for phosphatase,

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the rat cells have been readily demonstrated growing *in situ*, and this technique has thus provided further information on the transport and transplantation of heterologous hematopoietic cells.

Materials and Methods

Four groups of young (14–16 weeks of age) male LA F_1 [(C57L × A)F_1] mice were given whole-body exposure to X rays (250 kvp) at an LD100 dose level (810 r). The radiation factors and the exposure procedure were the same as those used previously (*10*). Approximately 1 hour later, each mouse received a single injection of a suspension of fresh isologous or heterologous bone marrow, as described previously (*4,5*). The amount of injected marrow and route of injection were as follows:

Group I—70 mg. rat marrow—intravenous
Group II—350 mg. rat marrow—intraperitoneal
Group III—1 mg. isologous mouse marrow—intravenous
Group IV—5 mg. isologous mouse marrow—intraperitoneal

The amount of bone marrow injected in each group of mice was based on survival studies described elsewhere (*2–4,11*), which had indicated that there was no technically feasible dose of intraperitoneally injected rat marrow that would result in survival to 15 days. The doses employed in the other groups had been shown to result in 15-day survival of approximately 65 percent in Group I, 90 percent in Group III, and 70 percent in Group IV.

A fifth group of mice, nonirradiated, were also injected with 70 mg. of rat marrow intravenously. A final group of mice were irradiated but received no injection, and all died within 12 days.

The mice used for the alkaline-phosphatase studies were sacrificed serially, by cervical dislocation, at approximately 2, 5, 7, 9, 12, and 14 days after irradiation. The tissues processed for phosphatase included: spleen with pancreas attached; thymus; lung; nodules of marrow growing in the peritoneal cavity; and a "button" of femoral bone marrow expressed onto lens paper by compressed air. The other femur was processed routinely and stained with hematoxylin and eosin in order to determine total marrow cellularity.

Two smears of blood, bone marrow, and peritoneal fluid were made on every animal at the time of sacrifice, and 1 of each type of smear was treated for alkaline phosphatase, as previously described (*5*). The other smears were routinely stained with Wright's stain.

In another series of experiments, mice injected intravenously with rat or mouse marrow were sacrificed at early times (2 min.–24 hrs.) for phosphatase studies on the lungs.

In all experiments, tissue sections and smears from normal mice of the same age, as well as from normal rats, were treated simultaneously for alkaline phosphatase for comparison.

Alkaline-Phosphatase Technique for Tissues

Fixation.—The tissues were fixed in cold (8° C.) 70 percent alcohol for 24 hours. Treatment in alcohol up to 96 hours produced only slight decrease in phosphatase activity.

After fixation, the tissues were transferred to 80 percent alcohol and allowed to come to room temperature (approximately 30 min.). Dehydration and paraffin infiltration were then carried out in the vacuum oven described by Roan and Gibbs (*12*), at approximately 380 mm. Hg, according to the following schedule:

Dehydration
Ninety-five percent alcohol—45 minutes
Absolute alcohol—45 minutes
Chloroform (C. P.)—45 minutes
Infiltration
Tissuemat (Fisher)—60 minutes
(m.p. 50–52° C.)

Sections were cut at 4μ, dried overnight at 37° C., deparaffinized in xylene, and run through several changes of graduated alcohols to distilled water.

Incubation.—The sections were then incubated in the glycerophosphate[6] substrate for 15 minutes at 37° C. The composition of the substrate and the further processing of the sections followed the procedure of Gomori as given by Cowdry (*13*).

Counterstaining was done with alcoholic eosin. Control sections were treated in the same fashion except for the omission of glycerophosphate from the substrate solution. Adjacent, or nearly adjacent, sections of all tissue blocks were stained routinely with hematoxylin and eosin for comparison with the slides treated for alkaline phosphatase.

Results

Normal Animals

In all the organs from normal mice examined, including spleen, pancreas, lung, thymus, and bone marrow, the only structure which uniformly stained intensely for alkaline phosphatase was vascular endothelium. Other structures that occasionally showed some positive phosphatase reaction under the conditions of these experiments included the periphery of the splenic follicles (figs. 1, 2), fragments of bone and cartilage trapped in the marrow "button," and the basement membrane of bronchial epithelium. With longer incubation in the substrate, up to 90 minutes, the splenic follicles stained brown throughout, making difficult the indentification of rat cells in the spleens of the experimental mice. Therefore, the incubation time of 15 minutes was used routinely. No

[6] Eastman Kodak Co., glycerophosphoric acid disodium salt (#644).

mouse blood cells, of either the myeloid or erythroid series, gave a positive phosphatase reaction in smears or in tissue sections.

The positive phosphatase reaction of *rat* blood and marrow cells in smears has been reported previously (*5,6,14*). Under the conditions of the present study (*i.e.*, 15-minute incubation), rat neutrophils in tissue sections stained black or dark brown; other hematopoietic elements of the rat stained only faintly, if at all.

Irradiated Mice

Group I (Rat Bone Marrow—Intravenous)

Spleen and bone marrow.—Two days after injection, occasional individual phosphatase-positive cells were present within the splenic pulp. The spleen and marrow sections stained with hematoxylin and eosin contained no foci of hematopoiesis.

By 5 days post irradiation, many small clusters of phosphatase-positive cells were present in the splenic pulp, and a few in the bone marrow. These coincided with foci of hematopoiesis noted in the hematoxylin-stained slides (figs. 3, 4).

Between 7 and 14 days post irradiation, the clusters of phosphatase-positive hematopoietic cells in the spleen and bone marrow increased progressively in size and number. The interfollicular-pulp space of the spleen became filled with actively growing hematopoietic cells of all types (figs. 5, 6), with eosinophilic myeloid elements, characteristic of rat marrow,[7] particularly prominent. Regeneration of lymphocytes within the splenic follicles lagged behind the growth of hematopoietic cells in the pulp, and no phosphatase-positive cells were present within the follicles. The increase in hematopoiesis in the bone marrow roughly paralleled that in the spleen, so that toward the end of the 2nd week after irradiation, the total cellularity of the marrow was approaching normal, with most of the cells showing intense phosphatase activity (figs. 7, 8).

Smears of blood and marrow, as previously reported (*5*), showed increasing numbers of phosphatase-positive cells after the 5th day post irradiation, concurrent with the growth of rat cells in the spleen and marrow cavity.

Despite the extensive growth of phosphatase-positive hematopoietic cells within the spleen, no such foci were ever found in the adjacent peripancreatic and mesenteric fat, nor in the pancreas itself. Smears of peritoneal fluid contained only a very occasional phosphatase-positive cell, appearing late in the 2nd week.

Thymus.—Regeneration of the thymus did not definitely begin until late in the 2nd week after irradiation, and then consisted of the return of the lymphocytic elements usually present and restoration of normal architecture. No foci of hematopoiesis or clusters of phosphatase-

[7] In the course of these studies it has been observed that rat bone marrow, growing either in the rat or in the mouse, usually contains clumps of intensely eosinophilic myeloid cells. Such clumps have not been noted in mouse bone marrow.

positive cells were observed in the thymus at any time (figs. 9, 10). Only a few individual positive cells located within blood vessels were observed (fig. 11), apparently representing rat neutrophils circulating in the peripheral blood.

Lung—Sections from mice sacrificed 2 minutes after injection of rat marrow contained numerous large and small emboli, consisting mainly of fibrous basophilic material and some clumps of intact cells. One hour after injection, the emboli were much less numerous and smaller in size, and many of the marrow cells were fragmented. By 24 hours, only an occasional small embolus remained, consisting of fibrin and a few degenerated cells. Subsequently, the lung sections contained only rare microscopic, organized thrombi, and there was no evidence of hematopoiesis at any time. At the end of the 2nd week, when the marrow was completely repopulated with phosphatase-positive cells, the only such cells noted in the lungs were a few individual leukocytes within blood vessels, such as were seen in the thymus.

In some irradiated mice injected intravenously with rat marrow the cells failed to "take," *e.g.*, these animals showed no growth of phosphatase-positive cells in any organs, and with this failure of hematopoietic "regeneration" gave evidence of increasingly severe radiation sickness. These mice undoubtedly represent the animals of this group, approximately 35 percent, that died within 15 days after irradiation.

Group II (Rat Bone Marrow—Intraperitoneal)

Spleen and bone marrow.—At 2 days post irradiation, as in the mice treated with rat marrow intravenously, there was maximum loss of lymphoid and blood-forming elements from the spleen and marrow, with no evidence of active hematopoiesis. No alkaline phosphatase-positive cells were noted *within* the spleen, but a few small clusters of positively stained cells were present in the adjacent peripancreatic fat, immediately *outside* of the splenic capsule.

Several large nodules of injected marrow were lodged at various sites in the peritoneal cavity, and these, on microscopic examination, contained clumps of viable, phosphatase-positive marrow cells mixed with necrotic debris. Smears of the peritoneal fluid were laden with phosphatase-positive cells, whereas blood and bone-marrow smears contained only very few cells with phosphatase activity.

At 5 and 7 days after irradiation, there was still no evidence of hematopoietic regeneration in the hematoxylin-stained slides of the spleen and marrow, and no foci of phosphatase-positive cells were seen in either organ. Lymphocytic regeneration in the splenic follicles was first noted on the 7th day. The intraperitoneal-marrow nodules continued to have a peripheral "shell" of viable, phosphatase-positive marrow, and numerous clumps of intensely stained marrow were growing in the mesenteric fat. In some cases, these were immediately adjacent to the capsule of the spleen (figs. 12, 13). Smears of the peritoneal fluid, blood, and bone marrow were similar to those taken at 2 days.

By the 9th day, numerous small foci of hematopoiesis were noted in the hematoxylin-stained sections of spleen and bone marrow (fig. 14); for the first time, several small clumps of phosphatase-positive cells were seen in the splenic pulp (fig. 15) and in the marrow. Comparison of the hematoxylin and phosphatase slides, however, showed that the majority of the hematopoietic foci found were negative for phosphatase activity. These observations indicate the *simultaneous* growth of both rat and mouse blood cells in the same organ. Within the peritoneal cavity, growth of rat marrow continued actively at the periphery of the injected nodules and in the mesenteric fat (fig. 16). The peritoneal fluid remained laden with strongly positive cells, and a slight increase in phosphatase-positive cells was observed in the blood and marrow smears.

After the 9th day, the intraperitoneally injected animals began to die with severe pneumonias and lung abscesses—characteristic terminal events of acute radiation sickness in these mice. The last survivor of the group was sacrificed *in extremis* on the 13th day after irradiation. Mice sacrificed during this period showed a continuing increase in total marrow cellularity and in lymphocytic and hematopoietic regeneration in the spleen. However, all these phases of regeneration lagged a number of days behind the *intravenously* injected mice, and the marrow cellularity of the last survivor was still only about 50 percent of normal on the 13th day.

Foci of phosphatase-positive cells within the spleen and marrow also increased in size and number between the 9th and 13th day (figs. 17–19) but remained only a fraction of the total hematopoietic regenerative process. Similarly, the number of phosphatase-positive cells in blood and marrow smears slowly increased but continued to represent a relatively small portion of the total population of neutrophils.

The findings within the peritoneal cavity remained as before. In the mouse sacrificed at 13 days, clumps of phosphatase-positive cells were noted within small intrapancreatic lymph nodes.

Thymus.—Throughout the period studied (13 days), the thymus showed only slight regeneration of lymphocytes, thus lagging several days behind the intravenously treated mice. No foci of hematopoiesis or of phosphatase-positive cells were noted within the thymus, although, during the 2nd week, occasional small clumps of strongly positive hematopoietic elements were observed in adjacent bronchial lymph nodes.

Group III (Mouse Marrow—Intravenous)

Spleen and bone marrow.—At no time, over the 2-week period studied, were any phosphatase-positive hematopoietic cells noted in the sections or smears examined, either as clumps or as individual cells. The pattern of hematopoietic regrowth, as observed in the hematoxylin-stained sections, was similar to that seen in the mice receiving *rat* marrow intravenously, except that in the isologous group it proceeded at a somewhat faster rate. By 5 days, hematopoiesis was well established in the spleen, and early regeneration of lymphocytes was also noted. These processes progressed

rapidly (figs. 20–23) so that by 10 to 12 days the total cellularity of the marrow had returned to normal, and the spleen contained extensive areas of hematopoiesis within the pulp, as well as well-formed lymphoid follicles. No marrow elements were observed in the peritoneal fluid or mesenteric fat at any time.

Thymus.—Thymic regeneration in this group was similar in pattern to that in Group I (Rat Marrow—Intravenous) but occurred somewhat earlier, with good lymphocytic regeneration present by the 9th day. No hematopoietic foci or phosphatase-positive blood cells were noted in any of the sections.

Lung.—As with the rat-marrow-treated animals, marrow emboli had nearly disappeared from the lungs by 24 hours post injection. Thereafter, only an occasional small organizing embolus was noted and there was no evidence of hematopoiesis, with or without phosphatase-positive cells.

Group IV (Mouse Marrow—Intraperitoneal)

Spleen and bone marrow.—No phosphatase-positive blood cells were observed in any of the smears or sections examined. Hematopoietic regeneration appeared earlier than in the Group II mice, treated with rat marrow intraperitoneally, despite the 70 times greater dose injected into the latter animals. By 5 days, the beginning of regeneration was noted in the spleen, and by 7 days, in the bone marrow. Although the growth of marrow elements at these sites continued to lag several days behind that in the Group III mice, it continued, thoughout the 2-week period studied, to be considerably more rapid than in the Group II animals. In table 1 are summarized the times to hematopoietic regeneration and to the appearance of clumps of phosphatase-positive cells in various organs in the different groups.

No nodules of marrow were visible in the peritoneal cavity, undoubtedly because of the relatively small amount of material injected, but the smears and sections revealed large numbers of phosphatase-*negative* marrow elements in the peritoneal fluid and growing in the mesenteric fat during the 2nd week after irradiation (figs. 24, 25).

TABLE 1.—*Comparative times of hematopoietic regeneration in X-irradiated (810 r) mice treated with rat or mouse marrow*

Material injected	Time to beginning of regeneration* (*days*)			Time to first phosphatase-positive clumps (*days*)			Time to complete regeneration* (*days*)
	Spleen	Marrow	Thymus	Spleen	Marrow	Thymus	Marrow
Rat marrow (IV).....	5	5	12	5	5	†	12–14
Rat marrow (IP).....	7–9	9	>13	9	9	—	>13
Mouse marrow (IV)..	5	5	9	—	—	—	10–12
Mouse marrow (IP)..	5–7	7	12	—	—	—	12–14

*Hematoxylin and eosin stain.

†Phosphatase-positive cell clumps never appeared.

Thymus.—Lymphocytic regeneration in the thymus began several days later than in the Group III animals and was not definitely established until the 12th day. Again there was no evidence of hematopoiesis or of phosphatase-positive cells.

Group V (Nonirradiated Controls)

From 2 minutes to 48 hours after the intravenous injection of rat marrow the number and distribution of emboli in the lungs was similar to those observed in Groups I and III. All the emboli contained phosphatase-positive cells. By 5 days, only an occasional bone spicule was noted in the lung sections as residual evidence of the injected marrow, and no phosphatase-positive cells were subsequently observed either singly or in clumps.

In the smears and sections of other organs, individual phosphatase-positive cells were noted for the first 2 days after injection, but none were observed thereafter, and there was never any evidence of growth of rat cells in the nonirradiated mice.

Discussion

The findings reported here indicate that rat marrow cells injected intravenously into irradiated mice are rapidly transported throughout the body. Lodging in the spleen and bone-marrow cavities, they divide and grow into recognizable foci of hematopoiesis within 5 days, The distribution and architecture of these phosphatase-positive foci, as well as the fact that they appear *only* in irradiated mice injected with heterologous marrow, would seem to establish definitely that they are indeed aggregates of growing rat cells. Within 2 weeks, the injected rat marrow repopulates the entire marrow cavity of the recipient mouse as well as extensive areas of the spleen. By supplying large numbers of new cells to the peripheral blood, the transplanted marrow apparently enables the host to combat successfully the ordinarily fatal acute radiation illness.

Although some of the injected rat cells initially lodge in the lung, all are apparently recirculated within 1 to 2 days, and there is no active growth of rat marrow at this site. Nor is there any evidence of the growth of rat cells in the thymus, and this organ lags behind the spleen and bone marrow in returning to normal proportions. Whether purely mechanical factors determine the sites of arrest and growth of injected marrow cells or whether the spleen and marrow cavity for some reason present a more favorable environment for hematopoiesis is not known. Coman (*15*) has demonstrated that, with respect to tumor emboli at least, the mechanics of circulation are the chief determinants of the sites of metastatic growth. On the other hand, it is noteworthy that, of the organs examined in the present experiments, only those which are normally sites of hematopoiesis in the mouse support the growth of intravenously injected rat marrow.

When injected intraperitoneally, however, the rat marrow grows readily in the loose tissues of the mesentery as previously observed by Congdon

and Lorenz (*3*). But it appears that only relatively small numbers of mature rat blood cells migrate from the peritoneal cavity into the peripheral blood. Previous work (*16*) has shown that hematopoietic regrowth must begin within 5 to 7 days after irradiation if recovery of the animal is to occur. If regeneration does not begin until 8 to 10 days, 100 percent mortality results, as in the case of mice receiving 800 r and no treatment or injected with isologous hematopoietic cells more than 3 days after irradiation (*17*). Therefore, the eventual appearance, at 9 days, of growing rat cells in the spleen and in the marrow cavity of the intraperitoneally injected mice occurs too late to be of benefit. Thus, it seems that irradiated mice injected intraperitoneally with rat marrow do not survive, primarily because of the failure of sufficient numbers of the mature rat cells formed in the peritoneal cavity to reach the peripheral blood and, secondarily, due to the fact that when *extraperitoneal* growth of rat marrow and regeneration of the mouse's own marrow finally does begin, at 9 days, it is too late to prevent death. It is of considerable interest, however, that in this group of mice, growing foci of rat marrow do eventually appear in the spleen, marrow and bronchial nodes, since it proves that significant numbers of cells capable of further division and growth can migrate spontaneously from the peritoneal cavity to other sites.

In comparing this group with the mice receiving *mouse* marrow intraperitoneally, a striking difference is noted. Although there was actually a greater quantity of marrow growing in the peritoneal cavity in the *heterologous* group, foci of hematopoiesis were noted 2 to 4 days earlier in the spleen and marrow cavity of the mice receiving isologous cells. Whether these foci in the isologous group represent autochthonous regeneration stimulated by a "humoral" factor from the intraperitoneal marrow or whether they consist of metastatic marrow cells from the peritoneal cavity we have no way to determine at present. However, the fact that hematopoiesis does begin at these sites 5 to 7 days after irradiation results in sufficiently early replacement of depleted blood cells to permit survival in over 50 percent of cases. Note, however, that all phases of regeneration in this group occur somewhat later than in the mice receiving isologous marrow *intravenously*, and consequently the percentage of survivals is lower in the intraperitoneally treated group.

The failure of the thymus to return to normal dimensions as rapidly as the spleen and bone marrow in all groups appears to be due, at least in part, to the complete absence of hematopoiesis, either autochthonous or *metastatic* within this organ. It is of interest that Brown *et al.* (*18*), in studies on *sublethally* irradiated mice, have observed no enhancement of regeneration of either the thymus or the spleen after injection of rat bone marrow, using the organ weights 25 days post irradiation as the criterion. On the other hand, Ford *et al.* (*8*), have reportedly identified intravenously injected mouse marrow in the thymus of homologous hosts.

In any event, it appears on histologic examination that, at least in mice receiving the very large doses of X irradiation used in the present experiments, the regeneration of *lymphocytes* in the thymus and elsewhere is as

dependent on the general condition of the animal as on the specific status of its hematopoietic system. Thus, even in some animals showing nearly complete marrow regeneration 2 weeks after irradiation, but also having severe pulmonary infections and concomitant weight loss, regeneration of the thymus failed to occur.

Summary

Gomori's histochemical technique for alkaline phosphatase was used to demonstrate the *in situ* growth pattern of rat marrow cells injected into irradiated mice. Rat bone marrow (70 mg.) injected *intravenously* appeared as foci of phosphatase-positive cells in the spleen and in the marrow cavity of the recipient mouse within 5 days. By the end of 2 weeks, the spleen and bone marrow were largely repopulated by rat-marrow elements which apparently provided enough new peripheral leukocytes to prevent death from acute radiation injury.

Rat bone marrow (350 mg.) injected *intraperitoneally* grew readily in the peritoneal cavity but did not appear as hematopoietic foci in the spleen and bone marrow until 9 days after injection. Irradiated mice receiving rat marrow intraperitoneally failed to survive for 2 weeks, apparently because insufficient new blood cells migrated from the peritoneal cavity into the peripheral blood and growth of marrow at other sites occurred too late to provide protection.

Following intraperitoneal injection of isologous bone marrow (5 mg.), hematopoietic regeneration in the spleen and in the marrow cavity of the host started within 5 to 7 days, and over half of these mice survived to 15 days. The mechanism is not clear, but in some way, isologous marrow elements, growing in the peritoneal cavity, resulted in more rapid hematopoietic regeneration at other sites than did heterologous marrow.

No hematopoiesis or clumps of phosphatase-positive cells were seen in the thymus after either intravenous or intraperitoneal injection of rat or mouse marrow, and under these circumstances the thymus always regenerated more slowly than the spleen and bone marrow of the recipient animals.

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PLATE 12

Sections on the *left* stained with hematoxylin and eosin; sections on the *right* treated for alkaline phosphatase and counterstained with eosin. All photographs × 3.

FIGURES 1 AND 2.—Normal spleen of LA F_1 mouse. *Note* intense phosphatase activity limited to vascular endothelium and faint activity at periphery of follicles.

FIGURE 3.—Mouse spleen 5 days after X irradiation (810 r) and intravenous injection of rat bone marrow. *Note* small, darkly stained foci of hematopoiesis.

FIGURE 4.—Same spleen. Many small clumps of phosphatase-positive (rat) cells in splenic pulp. Periphery of follicles well-stained.

FIGURES 5 AND 6.—Spleen 9 days after irradiation and intravenous rat marrow. Pulp completely filled by hematopoietic cells, the majority of which are phosphatase-positive.

FIGURES 7 AND 8.—Marrow "button" from the same mouse. Marrow solidly cellular, with intense phosphatase activity in most cells.

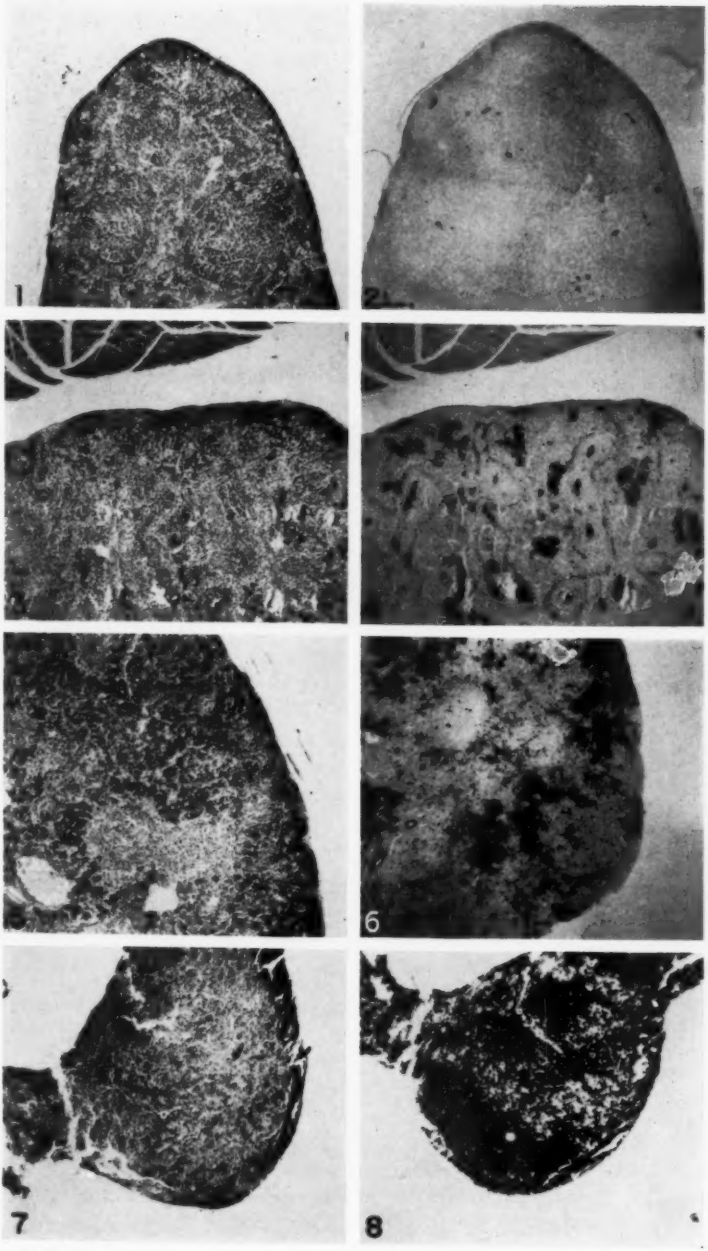

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PLATE 13

Figures 9, 12, and 14 stained with hematoxylin and eosin; all others treated for alkaline phosphatase and counterstained with eosin. All photographs × 32 except figure 11, which is × 100.

FIGURE 9.—Thymus 12 days after X irradiation (810 r) and intravenous injection of rat bone marrow. Some regeneration of lymphocytes with restoration of normal architecture. No evidence of hematopoiesis.

FIGURE 10.—Same thymus. Phosphatase activity limited to vascular endothelium.

FIGURE 11.—Higher power of same thymus. *Note* individual phosphatase-positive cells within vein.

FIGURE 12.—Spleen 5 days after X irradiation (810 r) and intraperitoneal injection of rat marrow. *Note* absence of hematopoiesis in spleen despite large clumps of marrow growing in adjacent peripancreatic fat.

FIGURE 13.—Same spleen. Clumps of phosphatase-positive cells growing external to the spleen; no such clumps within spleen.

FIGURE 14.—Spleen 9 days after X irradiation and intraperitoneal injection of rat marrow. *Note* numerous small, darkly staining foci of hematopoiesis.

FIGURE 15.—Same spleen. The small clump of cells adjacent to the intensely stained blood vessels is the only focus of phosphatase-positive blood cells noted in the section.

FIGURE 16.—Section from same mouse as in figures 14 and 15. Large clump of phosphatase-positive marrow growing in peripancreatic fat.

FIGURE 17.—Bone-marrow "button" from mouse femur 13 days after intraperitoneal injection of rat marrow. Several clumps of phosphatase-positive cells scattered in mass of phosphatase-negative marrow, suggesting simultaneous growth of rat and mouse cells.

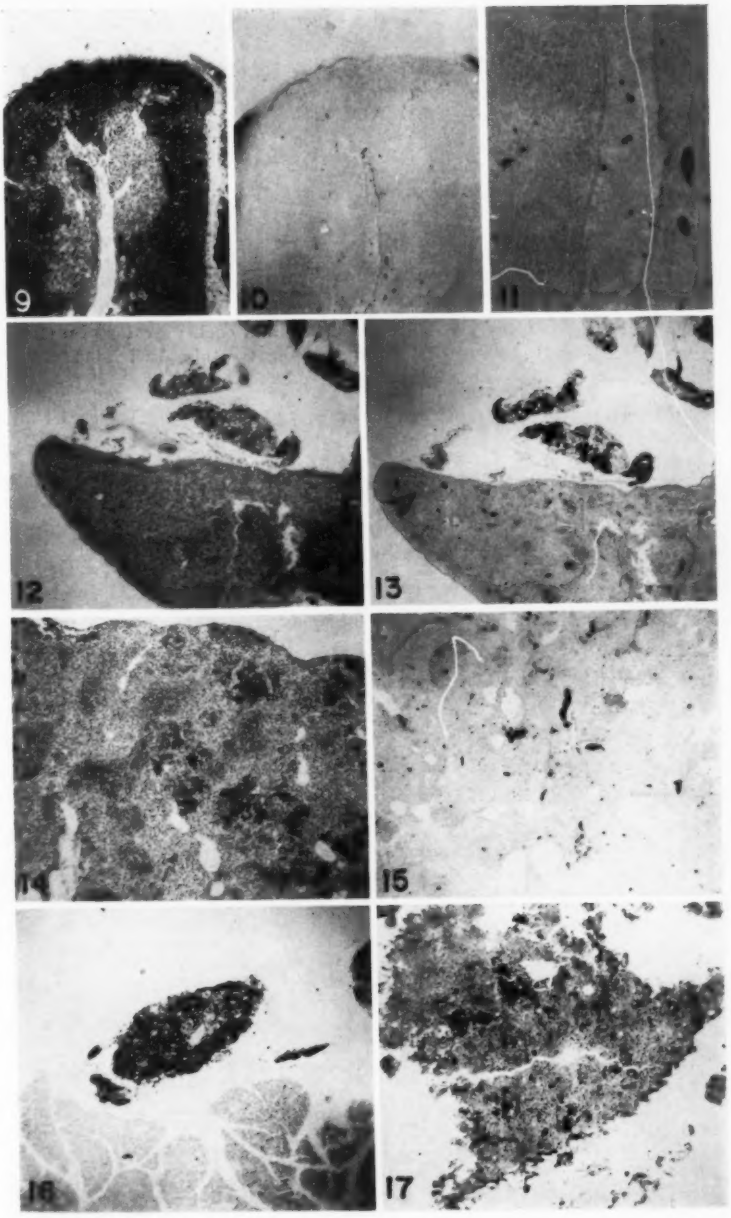
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PLATE 14

Sections on the *left* stained with hematoxylin and eosin; sections on the *right* treated for alkaline phosphatase and counterstained with eosin. All photographs × 32.

FIGURE 18.—Spleen section from same mouse as in figure 17. Pulp completely filled by hematopoietic cells.

FIGURE 19.—Same spleen. Numerous small foci of phosphatase-positive cells. Comparison with figure 18 suggests simultaneous growth of mouse and rat hematopoietic cells in same organ.

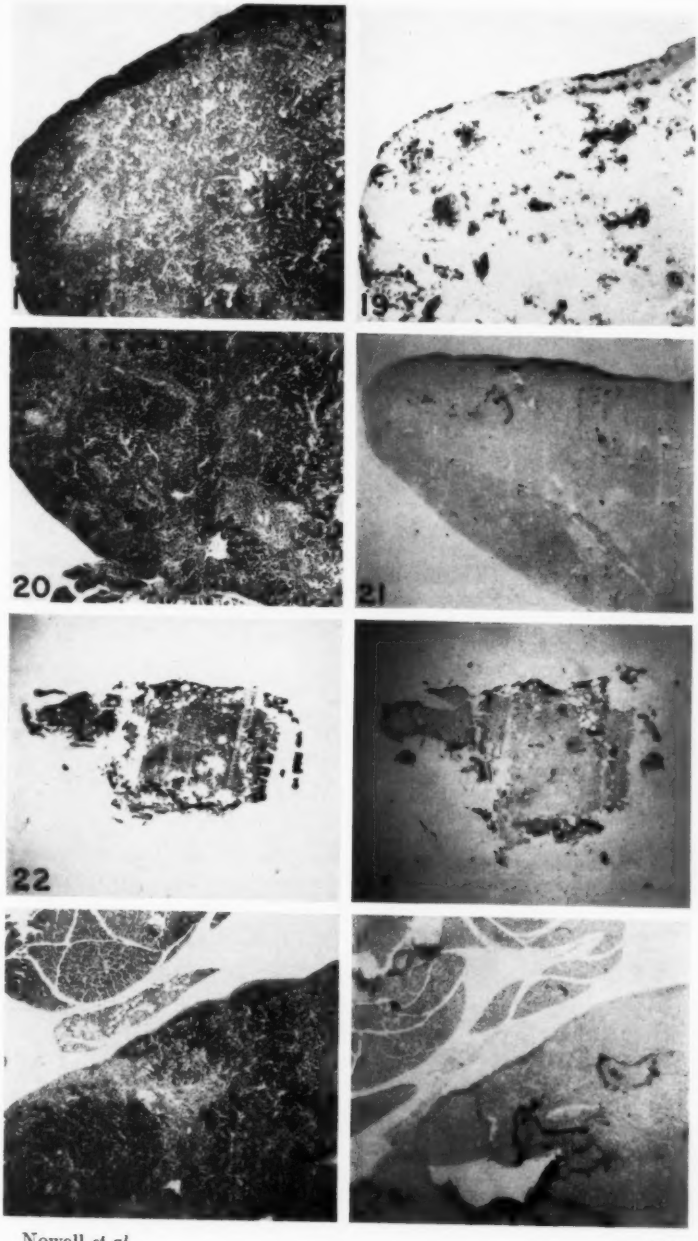
FIGURE 20.—Spleen 7 days after X irradiation (810 r) and intravenous injection of isologous mouse marrow. *Note* large areas of hematopoiesis.

FIGURE 21.—Same spleen, showing absence of phosphatase activity in hematopoietic cells.

FIGURES 22 AND 23.—Marrow "button" from same mouse, showing well-advanced regeneration. All cells phosphatase-negative. Dark areas at periphery of section are fragments of lens paper stained intensely by eosin.

FIGURE 24.—Spleen 7 days after X irradiation (810 r) and intraperitoneal injection of isologous mouse marrow. Large foci of hematopoiesis in spleen and in adjacent peripancreatic fat.

FIGURE 25.—Same spleen. Vascular endothelium and periphery of follicles strongly phosphatase-positive; intrasplenic and extrasplenic hematopoietic cells negative for phosphatase.



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Reduction of Synthesis of Diphosphopyridine Nucleotide in Tissues from Mice Bearing Transplantable Tumors [1]

V. S. WARAVDEKAR [2] *and* O. H. POWERS, *Laboratory of Chemical Pharmacology, National Cancer Institute,*[3] *Bethesda, Maryland*

It was reported previously (*1*) that the livers from mice bearing transplantable tumors showed a lowered capacity to synthesize diphosphopyridine nucleotide (DPN) than those from control mice. Similarly it was found that the DPN-synthesizing capacity of blood from mice diminished markedly soon after inoculation of tumor (*2*).

The present report deals with: *a*) the contrasting negative effect on the DPN-synthesizing enzyme of the blood observed after the transplantation of *normal* tissues; and *b*) observations pointing to the presence of an inhibitor for the DPN-synthesizing enzyme in the blood and tissues of the tumor-bearing animals.

Methods and Materials

Mice of the CA F_1 [(BALB/c $\times$ A)F_1] strain invariably were employed in these experiments, except where CDBA hybrids [(BALB/c $\times$ DBA/2)F_1] were used. The mice were inoculated, subcutaneously in the flank, with sarcoma 37, leukemia L1210, or with normal tissues such as liver, kidney, or spleen from the same strain; in a separate set of experiments sarcoma 37 was implanted in the tail. The amount of tumor or normal tissue transplanted as a mash was between 25 to 30 mg. per mouse.

The blood was drawn directly from the heart and was homogenized with distilled water. Liver, tumor, kidney, and spleen homogenates were prepared as before (*3*), and synthesis of DPN from nicotinamide mononucleotide [4] (NMN) was measured, as described previously (*1*).

Results

Sarcoma 37 in the flank.—In a previous communication (*2*) it was shown that at 24 hours after the inoculation of tumor the capacity of the blood to synthesize DPN was only about half that of the controls. In the present work, blood was withdrawn at short intervals after the implantation

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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] The authors gratefully acknowledge the cooperation of Dr. Louis Shuster of this laboratory in the preparation of this material.

TABLE 1.—*Synthesis of DPN by blood after subcutaneous inoculation of sarcoma 37 in the flank of CA F_1 mice*

Time after tumor inoculation (*minutes*)	Enzyme activity of blood* (*percent*)
5	100
15	73
30	68
35	57
60	45
120	55
180	44
240	51
360	48

50 ± 2 (S.E.)

*Blood from control mice (no tumors) synthesized 0.127–0.129 micromoles DPN per mg. N per hour.

Data in this column are the ratios of the blood-activity values for $\frac{\text{experimental mice}}{\text{control mice}} \times 100$.

Each value represents the activity of pooled blood from 2 animals and is the mean of 2 separate experiments.

of sarcoma 37 (*see* table 1). Within 1 hour after inoculation of tumor, the value dropped to about 50 percent of the control level. The enzyme activity remained at this level for at least 8 days following implantation of tumor in these experiments, in agreement with the findings reported (*2*) previously.

Sarcoma 37 in the tail.—Table 2 summarizes the results of an analogous series of experiments in which the tumor had been inoculated in the tail. As in the previous experiments in which the tumor was in the flank, the enzyme activity of the blood dropped sharply and rapidly and remained low for 9 days after tumor transplantation, at which time the experiment was terminated. On the 7th day of tumor growth, the tumors were surgically removed from 8 mice by severing the tails. Enzyme activity was

TABLE 2.—*Synthesis of DPN by blood from mice bearing sarcoma 37 in the tail*

Tumor-bearing mice	Time after tumor implantation (*days*)	Enzyme activity of blood* (*percent*)
Intact	1	59
	2	54
	3	55
	6	54
	7	51
	8	52
	9	49
Tail bearing tumor amputated on 7th day†	8	109
	9	104

*Blood from intact control mice (no tumors) synthesized 0.126–0.128 micromoles DPN per mg. N per hour.

Data in this column are the ratios of the blood-activity values for $\frac{\text{experimental mice}}{\text{control mice}} \times 100$.

†Seven days after implantation of the tumors, they were removed by amputating the tails. At the same time, tails were also removed from an equal number of control mice. Blood from the caudectomized control mice (no tumors) synthesized 0.121–0.127 micromoles DPN per mg. N per hour.

Each value represents the activity of pooled blood from 2 animals and is the mean of 2 sets of experiments.

measured in 4 of these the next day and in 4 others a day later. The data in table 2 show that the value rose rapidly to that of the controls within 1 day after caudectomy and was still normal the day following. The non-tumor-bearing mice, from which blood was drawn for comparative purposes on the 8th and 9th days, had also been caudectomized on the 7th day as a control on the effect of surgery on this enzyme activity.

Implantation of normal tissues.—Table 3 shows that subcutaneous injection of liver, kidney, or spleen, from other mice of same strain, did not affect the DPN-synthesizing capacity of the blood.

TABLE 3.—*Synthesis of DPN by blood from mice implanted with normal tissues*

Time after transplantation of tissues (*hours*)	Enzyme activity of blood* after implantation of:			
	Liver (*percent*)	Kidney (*percent*)	Spleen (*percent*)	Heart (*percent*)
1	96	—	—	—
4	95	103	98	103
24	99	96	98	102
144	92	98	97	104

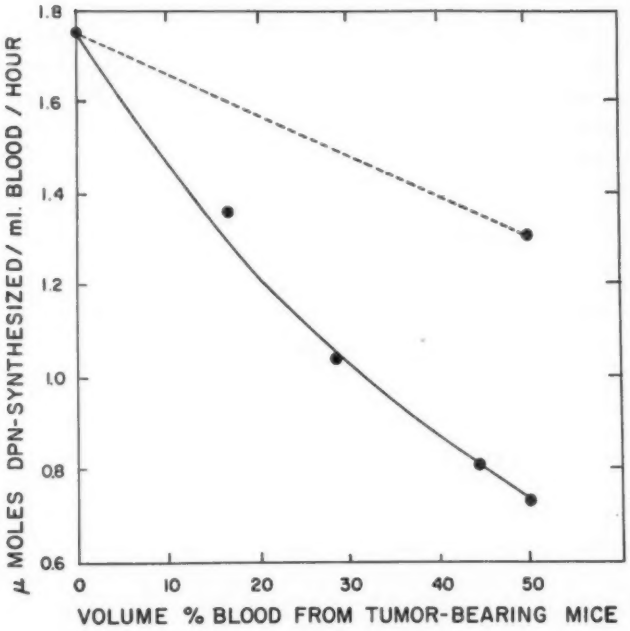
*Blood from control mice synthesized 0.122–0.130 micromoles DPN per mg. N per hour.

Data in this section are the ratios of the blood-activity values for $\frac{\text{experimental mice}}{\text{control mice}} \times 100$.

Enzyme inhibitor.—The pronounced drop in the capacity of blood to synthesize DPN, which is obvious as early as 1 hour after implantation of tumor, thus did not occur at all after analogous injection of normal tissues. This raised the question as to whether implantation of tumor is rapidly followed by the appearance, in the blood, of an inhibitor of the DPN-synthesizing enzyme. Accordingly, blood from tumor-bearing mice was added to that from controls to see whether any inhibition would be evident.

In a series of tubes containing 0.1 ml. (1:1 dilution) aliquots of pooled blood from control mice, pooled blood (1:1 dilution) from mice bearing 6-day-old sarcoma 37 was added in amounts which varied from 0.02 to 0.1, in increments of 0.02 ml. These were made up to a volume of 1.0 ml. with the reaction mixture, incubated for 10 minutes, and assayed for capacity to synthesize DPN. The results, summarized in text-figure 1, showed that the DPN-synthesizing activity of blood from control mice was reduced upon the addition of blood from tumor bearers. The activity of the mixture of equal proportion of blood from control mice with that from tumor bearers was reduced to about 56 percent of the calculated activity of the mixture.

The distribution of this enzyme activity between serum and red cells is shown in table 4. The serum was separated from clotted blood drawn from tumor-bearing and control mice. About 60 percent of the activity was found to reside in the serum and the rest in the clot. In this respect,



TEXT-FIGURE 1.—Reduction in synthesis of DPN by normal blood in mixtures with blood from mice bearing 6-day-old sarcoma 37.

The absolute values [for DPN formed per ml. (1 : 1 dilution) blood per hour] for the unmixed bloods were as follows:

Controls: 1.75 micromoles
Tumor bearers: 0.88 micromoles

Solid line represents the actual determination of the activities of the mixed blood, and the *broken line* shows the calculated activities for mixtures of blood from control and tumor-bearing mice.

TABLE 4.—*Synthesis of DPN by blood serum and red cells from control and tumor-bearing mice*

Blood component	Amount of DPN synthesized	
	Control mice* (*percent*)	Tumor-bearing mice† (*percent*)
Serum	60	59
Red cells	40	41

*Whole blood from control mice synthesized:—0.120 micromoles of DPN per mg. N per hr.
†Whole blood from tumor-bearing mice synthesized:—0.057 micromoles of DPN per mg. N per hr.

the distribution was the same whether tumor was present or not, even though there was a large difference between the absolute amounts of enzyme activity in the 2 types of blood. Results given in table 4 are the averages of 2 separate experiments.

Regressing tumor.—The DPN-synthesizing capacity of blood and of liver was altered soon after the implantation of sarcoma 37, which grew progressively. An experiment was then performed to compare the effect of inoculation of tumor tissue in 2 strains of mice, in one of which the tumor did not grow. Accordingly, L1210 was implanted in mice of the CDBA and CA F_1 strains. The tumors grew progressively, as usual, in the CDBA mice, and the animals died 8 to 9 days later. In the CA F_1 mice, the tumors did not grow in spite of the fact the tumor inoculum used was 3 times the dose employed for the CDBA mice; in CA F_1 mice sacrificed 1 month after tumor transplantation, only necrotic material was found at the injection site.

The data on the DPN-synthesizing capacity of the blood from these animals are summarized in table 5. In as short a time as 1 hour after implantation of tumor, the enzyme activity of the blood in both strains of animals was about 35 percent lower than that of the controls. However, 2 days later the activity had returned to normal in the CA F_1 mice, whereas in the CDBA mice it remained at the lowered level throughout the experiment. Thus the inhibitory factor was present in the blood of the animals while the tumor cells were active, but the inhibition disappeared with the death of the out-of-strain tumor cells.

Normal organs from tumor bearers.—Inasmuch as liver (*1*) and blood (*2*) from tumor-bearing mice have a lowered capacity to synthesize DPN, the observations were extended to spleen and kidney. Table 6 summarizes the results obtained with homogenates of liver, spleen, and kidney of CA F_1 mice with sarcoma 37. Enzyme activity in the liver and spleen

TABLE 5.—*Synthesis of DPN by blood from CA F_1 and CDBA mice inoculated with L1210*

Time after tumor inoculation (*hours*)	Enzyme activity of the blood*	
	Regression of implants in CA F_1 mice (*percent*)	Progressive tumor growth in CDBA mice (*percent*)
1	63	67
19	68	66
(*days*)		
2	95	62
3	93	71
4	97	68
5	95	54
7	108	67
8	97	64

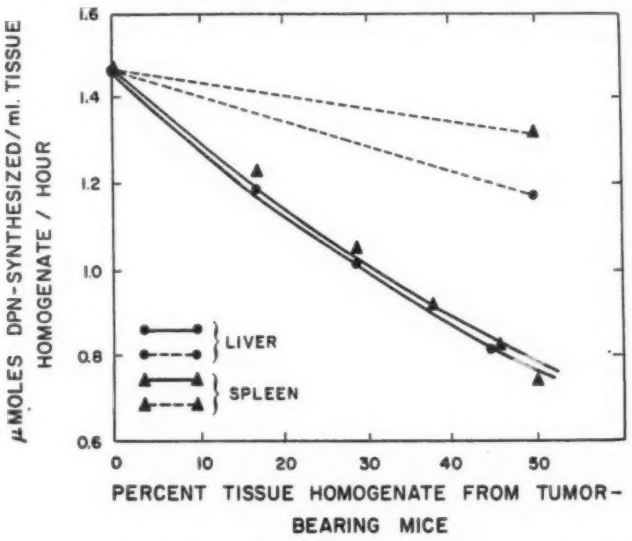
*Blood from CA F_1 control mice (no tumors) synthesized:—0.116-0.125 micromoles DPN per mg. N per hr.
Blood from CDBA control mice (no tumors) synthesized:—0.115-0.125 micromoles DPN per mg. N per hr.
Data in columns 2 and 3 are the ratios of the blood-activity values for $\frac{\text{experimental mice}}{\text{control mice}} \times 100$.
Each value represents the activity of pooled blood from 2 animals and is the mean of 2 separate experiments.

TABLE 6.—*Synthesis of DPN by homogenates of liver, spleen, and kidney from control mice and mice bearing 6-day-old sarcoma 37*

Tissue homogenate	Micromoles DPN formed per mg. N per hour		Percent of control value
	Control mice	Tumor-bearing mice	
Liver	0. 50	0. 30	60
Spleen	0. 50	0. 37	74
Kidney	0. 61	0. 56	92

was lowered to 60 and 74 percent, respectively, of control values, whereas the activity in the kidney dropped only slightly.

Homogenized liver from CA F_1 mice bearing sarcoma 37 was added to that from non-tumor bearers in various proportions; similar experiments were carried out with spleen homogenates (*see* text-fig. 2). Synthesis of



TEXT-FIGURE 2.—Reduction in synthesis of DPN by homogenates of liver and spleen from CA F_1 control mice upon addition of varying proportions of liver and spleen homogenates from mice bearing 6-day-old sarcoma 37.

The absolute values [for DPN formed per ml. (1:10 homogenate) per hour] for the tissue homogenates were as follows:

Control liver: 1.46 micromoles
Liver from tumor bearers: 0.88 micromoles
Control spleen: 1.46 micromoles
Spleen from tumor bearers: 1.17 micromoles

The *broken line* shows calculated activities for mixtures of homogenates of similar tissues from control and tumor-bearing mice.

DPN by liver from normal mice was reduced by addition of liver from tumor-bearing mice; entirely analogous results were obtained in the case of spleen. With both organs, the extent of reduction in enzyme activity was a function of the proportion of tissue added from the tumor bearers.

Discussion

Soon after the inoculation of tumor the rate of synthesis of DPN by the blood was reduced to half of the original level in the control mice. On removal of tumor the level was restored to normal. Inoculation of normal tissues such as liver, spleen, or kidney did not reduce the synthesis of DPN by blood.

In addition to liver and blood, spleen from the tumor-bearing animals also lowered the rate of synthesis of DPN; however, kidney showed only a negligible effect. The presence of progressively growing tumor gave rise to a humoral factor in systems such as blood, liver, and spleen of the host, which decreased the synthesis of DPN in these tissues. With the removal of tumor, the inhibitory humoral factor disappeared from the blood of the host, and the normal rate of DPN synthesis was resumed. Transplantation of L1210 into mice of the CDBA and CA F_1 strains yielded, in all of the former, tumors which grew until they resulted in the death of the mice, as expected, whereas in the latter, no progressively growing tumors were obtained, also as expected; those tumors which started to grow in the CA F_1 mice later regressed, and the animals lived indefinitely. The blood from both strains of mice showed a lowered capacity to synthesize DPN 1 hour after the inoculation of the tumor. However, by 48 hours the blood from CA F_1 mice showed a normal rate of DPN synthesis, whereas the blood from the CDBA mice maintained a lowered DPN-synthesizing capacity throughout the experiment until the animals died. Thus the reduction of the enzyme activity of the blood ran parallel with the presence of viable, proliferating tumor cells. The enzyme activity returned to normal with death of the out-of-strain tumor cells.

The results from *in vitro* studies on the inhibition of DPN-synthesizing enzyme in tissues from control mice by the addition of tissues from tumor-bearing mice are consistent with the view that sarcoma 37 gave rise to a humoral factor, which, in turn, had an inhibitory effect on the DPN-synthesizing enzyme of the tissues.

Further work on the fractionation of blood from control and tumor-bearing animals and on the effect of incubation of the mixtures at various intervals to study the changes in the synthesis of DPN is in progress.

Summary

1) The synthesis of DPN by blood from mice, 1 hour after intramuscular implantation of tumor, was lowered to about 50 percent of that of the controls.

2) Inoculation of tumor in the tails of animals lowered the capacity of blood to synthesize DPN to 50 percent of that of the controls. The

DPN-synthesizing capacity returned to normal soon after surgical removal of the tumor.

3) Implantation of normal tissues into mice produced no effect on the synthesis of DPN by blood.

4) Inoculation of leukemia L1210 into CDBA and CA F_1 strains of mice produced tumors in CDBA mice but not in CA F_1 mice. The capacity to synthesize DPN by blood from CDBA mice bearing leukemia was reduced to about 65 percent of the control value. The synthesis of DPN by blood from CA F_1 mice was also reduced to 63 percent of that of controls soon after the inoculation of tumor but, after 48 hours, the DPN-synthesizing capacity of blood returned to normal.

5) Like blood and liver, spleen of the tumor-bearing animals possessed a lowered rate of DPN synthesis. However, kidney showed almost the same rate of DPN synthesis as that of the controls.

6) *In vitro* experiments on the inhibition of the DPN-synthesizing enzyme of blood from control mice by the addition of blood from tumor-bearing mice indicated the presence of a humoral factor in the blood of tumor-bearing animals. Likewise, the synthesis of DPN by homogenates of liver or spleen from control mice was inhibited by the addition of homogenates of liver or spleen from tumor-bearing animals.

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ANNOUNCEMENTS

SEVENTH INTERNATIONAL CANCER CONGRESS

LONDON, ENGLAND

July, 1958

Sponsored by

The International Union Against Cancer

The Seventh International Cancer Congress will be held in London, England, July 6 to 12, 1958, under, the Presidency of Sir Stanford Cade. Congress headquarters will be The Royal Festival Hall.

There will be two main sessions of the Congress:

A. Experimental
B. Clinical and Cancer Control

Special emphasis will be placed on Hormones and Cancer, Chemotherapy, Carcinogenesis, and Cancer of the Lung.

Proferred papers will only be considered if submitted with an accompanying abstract (not over 200 words) before October 1957 and if dealing with new and unpublished work.

The registration fee for the Congress will be £10 (ten pounds) or $30 (thirty dollars) and the latest date for registration without late fee will be *January 1, 1958.*

Registration forms and a preliminary program will be available early in 1957 on application to

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Symposium on Fundamental Cancer Research

The Eleventh Annual M. D. Anderson Hospital and Tumor Institute Symposium on Fundamental Cancer Research will be held March 7, 8, and 9, 1957, at The University of Texas M. D. Anderson Hospital and Tumor Institute in the Texas Medical Center, Houston.

The general topic for the Symposium is "Viruses and Tumor Growth."

Fourth International Gerontological Congress

Scientists and others with experience in fields associated with the various aspects of aging will meet at the Fourth International Gerontological Congress in Merano, Italy, on July 14, 1957.

All abstracts of papers to be presented at this Congress must be submitted to the North American Committee of Cooperation for review before acceptance. The Chairman of this Committee is Dr. E. V. Cowdry, Washington University School of Medicine, 660 South Kingshighway, St. Louis, Mo.

The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

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2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

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